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Perspectives in Cancer Research

Staging and Treatment of Hodgkin's Disease

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THE FIGHT against illness is like any other kind of warfare, inasmuch as it comprises a period of open conflict, with spear-head thrusts and sweeping advances, followed by a phase during which control over conquered territory has to be consolidated.

We believe this phase has now been reached for Hodgkin's disease. Recent years have seen revolutionary progress in the approach of the illness and its treatment: noteworthy developments include the use of lymphography [1], the use of new histological classifications [2, 3] and the practice of laparotomy accompanied by splenectomy and ovary transposition [1, 4, 5]. In the field of radiotherapy, new irradiation techniques have been introduced with more penetrating sources, higher doses, for wider targets with lymphnode areas anatomically or physiologically adjacent, to the lesions [6, 7]. Progress in chemotherapy includes new effective drugs such as periwinkle alcaloids and procarbazine, as well as new methods of administering and combining drugs, and combination of radio and chemotherapy [7–9]. All this has radically changed the picture as regards Hodgkin's disease and its treatment.

A feeling of uneasiness, however, is beginning to mar the general satisfaction at these successes, for which no cancerologist of the preceding generation would have dared to hope. For some people consider that the accumulation of procedures, improvements, and even minor trimmings, are beginning to give the clinical picture of Hodgkin's disease a somewhat over-elaborated and overloaded look that does not simply detract from its elegance but undermines its soundness. Patients are cured twice over (by radiotherapy and chemotherapy) which may sometimes be once too much. For chemotherapy aplasias, unpleasant after effects and serious complications of radiotherapy may even prove fatal, and such patients, who are supposed to have been cured, may be left with extensive sclerosis, mediastinitis, pericarditis, post-radial myelopathia, castration in women, azospermia in men, secondary malignant diseases, epithelioma, sarcoma and even leukemia [7, 9]. All this calls for a more appropriate economy of means in effecting cures and for more comfort for the patients once they have been completed.

Some of the practices used to combat Hodgkin's disease are necessary, but others are questionable, either on account of their gravity or because their interest is uncertain.

The necessary medical acts need only to be listed here briefly: they include histological biopsy, indispensable for diagnosis, anamnesis and an effort to ascertain whether the patient is suffering or has suffered from fever, noteworthy loss of weight or intensive sweating. Clinical and radiological surveys are also necessary, including X-rays of the thorax, inclined frontal tomographies and bilateral lymphography, as biological test, no more sophisticated test has yet been able to replace the sedimentation rate.

The decision considered controversial on account of its gravity is laparotomy with splenectomy [1, 4, 5, 7, 10].

Few problems appear to have been tackled in a more irrational manner, yet the motives for such action are simple and logical: lymphography cannot explore the entire lymphnode regions in the abdomen, the interpretation of liver and spleen scintigraphy is problematical and there is not even any correlation between the size of the spleen and its penetration by the disease. For all these reasons, exploratory laparotomy and splenectomy, with all the successive lymphnode liver and bone biopsies they imply, are necessary to obtain a complete picture of the subdiaphragmatic stage of Hodgkin's disease [7]. Yet laparotomy involves delaying essential

therapy, as well as a certain rate of morbidity or even mortality (a mortality rate, however low, is inevitable in abdominal surgery) and systematic removal of so-called normal spleens, all this in relation to patients whose immunological balance is already disturbed. Some people believe preliminary laparotomy has such obvious advantages that they can no longer conceive of treating Hodgkin's disease without it, whatever the clinical stage of the case (Kaplan thinks this even holds for Stage IV, which seems a little extreme); others feel the drawbacks and risks of laparotomy so keenly that they refuse to perform it at all.

Such confusion is normal during the years of trials that follow the introduction of a new technique, but practical experience usually clarifies the situation. Unfortunately this has not been the case for laparotomy in Hodgkin's disease and the position is still confused. The practice of laparotomy has chiefly led to negative conclusions, making it clear, for instance, that spleen size and splenic scintigraphy do little to make prediction of its Hodgkin infiltration, that the spleen may simply be large for "reactional" reasons and that, in particular, the clinical and histological staging do not permit reliable forecasts of splenic conditions [4, 5, 7]. True, the more advanced the prelaparotomy staging the more likely the spleen is to be positive, and in Stages I and II splenic infiltration does indeed seem more common where histological type is bad (mixed cellularity and lymphocyte depletion). All the same, in Stages I and II and therefore, in prelaparotomy staging with only upper diaphragmatic lesions, about 20% of spleens are positive even when histological type is good (lymphocyte predominance or nodular sclerosis [1, 4, 5, 7]. Many consider that this solves the problem, since such a high spleen lesion rate, unexpected, under what appear to be the most favourable conditions, makes it imperative to take systematic action with regard to the spleen, and, if it is not removed, it will have to be irradiated. But the problem is not so simple: to disregard spleen lesions in 20 out of 100 patients during the initial treatment does not mean that they are lost cases. One does not know how microscopic lesions would develop. Let us assume that they would all tend to become "visible". In such cases a second course of treatment, centred on splenectomy, ensures a high percentage of long remissions and probable cures, for cases with "good histology". What are the effects of splenectomy or spleen irradiation on the other 80 patients whose spleen is "negative"? The basic problem is in fact to know, for Stages I and II, if systematic initial laparotomy and splenectomy lead, in the "short run" (roughly the duration of the first remission) and, even more, in the "long run" (duration of survival) to better, comparable or worse results than those obtained if laparotomy is not systematic (splenectomy being performed later, should any splenic lesions develop). Although it is generally acknowledged that initial laparotomy and splenectomy helped to improve our understanding of Hodgkin's disease [1, 4, 5, 7], it is not certain that they improve patients' life expectancy.

The results of a trial conducted along these lines might vary according to the presentations of the disease. Oddly enough, however, in this era of randomized, cooperative and international trials, such a trial has never been carried out, or even initiated. The only choice left to the therapist is removal of the spleen or irradiation of the splenic region, with the obvious drawbacks this involves for the left kidney and left costal diaphragmatic sinus. Systematic laparotomy accompanied by splenectomy is therefore tending to become generalized for Stages I, II and IIIA, regardless of histological types, which is reasonable in the light of present results. But the matter is unlikely to end there, and a greater variety of indications will no doubt gradually come to light. However, as long as no trials are conducted of the kind described above, this regrettable deficiency will make the process long and difficult.

On top of all this, case histories of Hodgkin's disease have generally been enriched or encumbered by investigations the practical contribution of which to progress has not always been obvious. For instance, the illness seems to attract all the new techniques in radiology. The various kinds of arteriography and cavography are not to be used in this disease, liver and spleen scintigraphy can be useful, provided one remembers that they lead to extremely uncertain conclusions. As regards the radiological aspects of the illness, we believe that in recent years technetium pyrophosphate bone scintigraphy, a remarkably sound technique, is the most outstanding progress.

Biologists, and above all immunologists, seem even more fascinated by Hodgkin's disease than radiologists. No research group that has initiated work on immunological tests has failed to start off by initiating a programme for the study of Hodgkin's disease. At one time, it seemed probable that immunologists, like clinicians and histologists, would succeed in establishing a classification system for Hodgkin's disease which would lead to a prognosis and help to determine the treatment prescribed. However this hope has

not materialized [11] and it cannot be said that even the most sophisticated immunological surveys have added much to our understanding of Hodgkin's disease, over and above the initial observation of frequent tuberculin anergy.

The immunologists have certainly not said their last word and will, no doubt, succeed in working out more instructive tests than those currently in use. However, even though this type of research should continue, nothing, if the patients' interests alone are considered, can, at the present time, justify any delay whatever in initiating the treatment to perfect the immunological survey.

In our present knowledge of Hodgkin's disease, a good staging [1, 5] can be simple, and even sparing of investigations.

Although the procedures involved in drawing up staging of Hodgkin's disease sometimes seem cluttered up with superfluous investigations, that this is even truer as regards treatment, which is positively inflationary, with radio and chemotherapists jostling each other in their eagerness to look after these patients, who are actually curable.

Despite the progress in this sphere during the past 20 yr, complete agreement has never been reached on any one form of the illness. Nevertheless a few well-established facts are now formally recognized and we believe they ought to guide our present choices. In Stages I, II and IIIA of Hodgkin's disease, permanent cures can be obtained by radiotherapy [6, 7]. The doses are well codified—4000 rad in 4 weeks; treatment should be directed at a large field that takes in affected lymph node areas and the areas adjoining them. Upper diaphragmatic lesions require mantle irradiation.

More recent trials showed that either splenectomy or irradiation of the splenic region, as well as systematic irradiation of the lumbaraortic lymph nodes, one month after mantle irradiation, considerably improve longterm results for upper diaphragmatic lesions of Stages I and II. Where the histology of such cases is poor (mixed cellularity and lymphocyte depletion) the results of irradiation alone can be improved by a second treatment comprising systematic chemotherapy, provided it extends over a prolonged period of 18 months to 2 yr and is well tolerated, as is the case for prolonged vinblastine chemotherapy [6]. IIIA stages require mantle irradiation, followed by inversed γ-irradiation after a month's interval; there again, secondary extended chemotherapy seems helpful.

Discussion regarding treatment of these stages centres on the possible interest of applying chemotherapy before radiotherapy. Encouraging results have been reported by this method [8] but there is of course the risk that such chemotherapy, designed to improve the essential means of treatment—radiotherapy—may jeopardize the latter's effect by imposing limitation of dosage; there is also the danger that even when the treatment is well tolerated it may exceed what is necessary to effect a cure, and this might have long term consequences which are still difficult to assess, such as azoospermia or malignant secondary disease.

Another established fact is the remarkable effectiveness of combined chemotherapy in Stage IV cases: thus, 80% complete remissions were obtained using the MOPP Protocol (nitrogen mustard, vincristine, procarbazine and prednisone) or its derivatives [9, 10]. Long term results are not perhaps as good as expected, since delayed relapses were frequent.

The problem is to find out what can be done after the conclusion of MOPP treatment, which in principle lasts only 6 months. It would be possible to continue courses of MOPP and lengthen the intervals between them but in practice this could involve a risk of lasting aplasia, perhaps due in the main to the repeated doses of nitrogen mustard. Often, the only possible treatment after MOPP is chemotherapy with one compound having little myelotoxicity, such as the administration of vinblastine over a prolonged period.

Stage IIIB cases ("B" signs are fever, 10% loss of wt and sweating at night) are probably those that involve the largest number of poorly solved problems; moreover it is in this field that chemo- and radiotherapists find it most difficult to delineate their respective spheres of action.

In practice, some IIIB cases are "big" IIIB cases, with high fever, drastic loss of wt and alarming general condition. Some are "little or minor" IIIB cases with a light and passing fever or occasional night sweating.

It seems clear to us that treatment of "big" IIIB cases should start with chemotherapy. Indeed it would take an extremely unrelenting radiotherapist to push a patient shivering with fever under high-powered irradiation devices [7]. The results of MOPP-type chemotherapy, or its derivatives, have proved excellent [8, 9, 10]. However it remains to be seen whether chemotherapy alone can be relied on to cure such cases.

Some use only chemotherapy, full course of MOPP followed by whatever long term chemotherapy remains possible. Some after a chemotherapy, for instance 3 courses of MOPP, will attempt eradication radiotherapy, followed by a second course of chemotherapy, according to the

hematological tolerance of the patient, 3 courses of MOPP if it is good, vinblastine only if it is poor.

In the case of so-called minor IIIBs there will obviously be a tendency to use the second method of successive chemo- and radiotherapy; one might even start with eradication radiotherapy and follow it up systematically with chemotherapy over a prolonged period.

Trials provide the only means of elucidating these questions, although this will not be easy because the concept of IIIB is so much clearer in theory than in practice.

To sum up the present position, it seems reasonable to suggest the following clinical procedures for treating Hodgkin's disease: for Stages I, II and IIIA—laparotomy accompanied by splenectomy; after that, cases remaining in Stages I and II may be treated by mantle field radiotherapy followed by lumbar-aortic radiotherapy; "bad" forms of such cases (poor histology or over 40 yr old) should then be subjected to extended chemotherapy, for instance one and a half or two yr vinblastine treatment. Stage IV should be given MOPP-type chemotherapy followed by extended chemotherapy, the most effective and easiest form to handle being vinblastine treatment. A major IIIB case should either be treated like a Stage IV or be given several courses of chemotherapy (for instance, 3 series of MOPP) followed by eradication radiotherapy and extended vinblastine chemotherapy. This procedure could also be applied to minor IIIB cases, the very slight IIIBs being treated like IIIAs.

Hodgkin's disease has occupied the limelight for 20 yr now in the cancer field and has perhaps suffered from being in the forefront for so long. Some patients seem positively overburdened by all the different protocols controlled by immunologists, surgeons, radiotherapists and chemotherapists. However, since much has now been done for this disease, a great deal of attention and enthusiasm will now be switched to other malignant diseases, and this will create a calmer climate in which to effect the painstaking adjustments still necessary to bring Hodgkin's disease under more complete control.

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Biochemical and Clinical Studies of Chronic Lymphocytic Leukemia*

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Abstract—*Attempts were made to identify biochemical parameters that would characterize leukemic cells obtained from patients with chronic lymphocytic leukemia at early stages of the disease and with more aggressive phases. These studies included (a) the quantitative estimation of the ribonucleotide pool and of the ability of the cells to utilize* de novo *vs salvage metabolites; and (b) the measurement of the extent of metabolic activation of selected antimetabolites such as ara-C and 6-MP. Cells obtained from the peripheral blood of six male patients with chronic lymphocytic leukemia were shown to possess a reduced ability to utilize* de novo *pathways of purine and pyrimidine biosynthesis as indicated by the lack of ATP and UTP formation from glycine and orotic acid, respectively. In one of these patients, however, although the cells initially lacked the ability of utilizing glycine and orotate, a progressive increase in TdR and glycine incorporation into nucleic acid was observed, and was found to reach a plateau within two months prior to death. In another patient, the ability of cells to utilize glycine for the formation of ATP was found to be coincident with the appearance of advanced and aggressive disease. Concurrent with these shifts from total reliance of cells on salvage pathways for nucleic acid synthesis, to at least partial dependence on* de novo *pathways, a change in the ribonucleotide profile was observed with a shift from lower to higher ATP/IMP ratios. In the four other patients studied, the clinical and the biochemical profiles were both stable and unchanged to date.*

INTRODUCTION

CHRONIC lymphocytic leukemia (CLL) is a disease which frequently evolves from a benign lymphocytosis to a clinically serious disorder, characterized by problems such as obstructive lymphadenopathy and anemia, thrombocytopenia, granulocytopenia, cutaneous disorders and defects in humoral immunity [1]. Since the disease usually runs a protracted course, it is not uncommon for patients to become refractory to conventional therapy at a time when they remain in otherwise relatively good clinical condition. The inability to offer further effective therapy at this stage results in the gradual deterioration and eventual death of the patient from disease or intercurrent complications.

At various times after diagnosis, most patients with CLL develop increasingly aggressive disease. Various staging systems have shown some correlation with survival [2, 3]. Whether these different stages represent points in the natural history of the disease or subtly different disease types has not yet been clearly determined.

As a first step towards the rational management of patients refractory to conventional therapy, biochemical parameters possibly relevant to drug action, may be measured and therapeutic agents may be logically chosen on the basis of measurements of biochemical parameters *in vitro*. For example, recently a study by Scholar and Calabresi showed that the enzymes necessary for the *de novo* synthesis of purines were low in lymphocytes from both normal donors and patients with CLL [4]. Thus, inhibitors of *de novo* purine biosynthesis would not represent a logical choice in such cases.

This report describes a detailed biochemical and clinical study of 6 patients with CLL at various stages of their disease, including 2 patients who underwent clinical and biochemical alterations of the disease.

MATERIAL AND METHODS

Material

Ribonucleoside mono-, di- and tri-phosphate standards and Ficoll (mol. wt approx. 400,000)

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were obtained from Sigma Chemical Co. (St. Louis, MO). All phosphorylated compounds were the disodium salts of the 5′ derivatives and were dissolved in 2 mM ammonium formate buffer, pH 4.4. Sodium diatrizoate was obtained from Winthrop Laboratories (New York, NY). Glycine-2-^{14}C, 36.7 mc/mmole; adenosine-8-^{14}C, 52 mc/mmole; and arabinosyl cytosine-5-^{3}H, 10–16 C/mmole were obtained from Schwarz/Mann (Orangeburg, NY). Thymidine-2-^{14}C, 56 mc/mmole was from Amersham and Searle (Arlington Heights, Ill.). 6-MP-8-^{14}C, 0.25 mc/16.7 mg was obtained from New England Nuclear (Boston, Mass.). Orotic acid-5-^{3}H, 7 C/mmole was from Nuclear Dynamics, potassium phosphate reagent was obtained from Baker Chemical Co., (Philipsburg, NJ).

Cell separation

(a) *Leukapheresis.* Continuous flow leukapheresis with an IBM–NCI cell centrifuge was used on several occasions to obtain lymphocyte blood fractions for study. Patients were premedicated with 5000 heparin i.v. after outflow and return catheters had been introduced into the antecubital veins. ACD solution, formula A, American Instrument Co., 1 ml/40 ml of blood was infused into the extracorporeal circuit before centrifugation. The centrifuge bowl was run at 800 rev/min. In normal individuals this procedure yields approximately $5–10 \times 10^9$ white blood cells (80–90% lymphocytes) in 2–3 hr [5]. In leukemic patients, the actual yield depends on the composition and quantity of the leukocyte fraction in the peripheral blood.

(b) *Density gradient cell separation.* Hypaque–Ficoll solutions were prepared as follows: (34%), w/v sodium diatrizoate (10 parts) and 9% w/v Ficoll (24 parts) were mixed. Solutions were kept in the dark, frozen and thawed out prior to use [6].

The first 10–15 ml of white cell enriched blood separated by leukapheresis was overlaid on 8 ml of a Hypaque–Ficoll gradient in a 15 ml conical centrifuge tube with 8 ml of cell suspension. Tubes were then centrifuged at $600 \times g$ for 25 min at room temperature. Three distinctly separate areas were observed. Uppermost was plasma; in the middle, lymphocytes; and at the bottom of the tube, granulocytes and red blood cells. Lymphocytes were removed, washed with RPMI-1640 and counted. The entire procedure required about 45 min. Differential counts were made on the cell suspension, after leukapheresis and Hypaque–Ficoll separation fractions using Wright–Giemsa stained slides.

Ribonucleotide separation

Lymphocytes obtained from CLL patients were extracted with 6% perchloric acid (10^7 cells/0.2 ml) and centrifuged at $2000 \times g$ for 1 min. The acid soluble fraction was then immediately adjusted to pH 7.0 with 2 N potassium hydroxide and the precipitate was removed by centrifugation. The acid-soluble fraction was immediately analyzed for its ribonucleotide content using either a DEAE cellulose column [7] or a high pressure liquid chromatograph.

A Dupont high pressure liquid chromatograph, Model 830, fitted with an ABX column (2 m × 2 mm, U-shaped) and with a dual cell detector was used for high pressure liquid chromatography (HPLC) separation of the ribonucleotides. Separation was carried out using a concave gradient No. 2. The initial buffer concentration was 2 mM potassium phosphate (pH 3.0) and the final was 0.5 M (pH 4.4), with a gradient of 3% per min. This method was used for the separation of ribonucleoside mono-, di- and tri-phosphates as well as the various ara-C metabolites, ara-CMP ara-UMP, ara-CDP and ara-CTP. HPLC separation was carried out at room temperature under a pressure of 1000 psi resulting in a flow rate of about 1 ml/min. The effluent was monitored simultaneously at 254 and 280 nm with absorption recorded on a 2-pen Hewlett–Packard recorder. Areas under each peak were automatically integrated using an Autolab Minigrator. In experiments in which drug metabolism was investigated, the effluents representing individual absorption peaks were collected directly into counting vials. Radioactivity in each vial was determined using a Packard Model 330 liquid scintillation spectrophotometer.

Metabolism of metabolites and antimetabolites

Separated and purified lymphocytes (5.0×10^7 cells/7 ml) were incubated at 37°C for 30 min with labeled agents (20 uCi of ara-C-5-^{3}H, 5 uCi each of 6-mercaptopurine (6-MP), adenosine ribonucleotide (AR), thymidine deoxyribonucleotide (TdR) and glycine. At the end of the incubation period, tubes were centrifuged at $600 \times g$ for 2 min, washed twice with a 0.9% sodium NaCl and a semi-dry pellet was either taken up directly into a counting vial for the measurement of total uptake of the labeled agent or extracted with 6% perchloric acid (PCA) for the analysis of acid-soluble fraction by HPLC. Incorporation of labeled agents into RNA and DNA was determined as described previously. Each vial was counted at least twice for 10 min each time.

Patients

All patients studied were considered to have classical chronic lymphocytic leukemia based on standard clinical and cytologic criteria. The clinical characteristics of the patients are summarized in Table 1. At the time of study, all patients except PM were under chemotherapeutic treatment and all were stage III or IV.*

Two patients, VL and GF, presented with uremia and hyperuricemia [3] while the other 4 patients had minimal symptoms at presentation. One patient, PM, had been under observation for a period of 11 yr during which time the disease evolved from stage II to stage IV.

One patient, PM, is described in detail. This 56-yr-old Caucasian male was incidentally discovered to have CLL during a routine yearly examination in 1962. In 1963 he developed adenopathy, primarily in the central region, and increasing lymphocyte counts. When the count was 70,000, he was treated initially with chlorambucil and then with tetraethylmelamine (TEM) intermittently for 8 yr until he became refractory to this agent. For an additional yr he was treated with very modest success but with progressive problems of anemia, using chlorambucil and high doses of prednisone. Increasing adenopathy and rising lymphocyte counts together with worsening thrombocytopenia occurred and he was referred to this Institute. In the early part of 1974, the patient was treated with various regimens utilizing cyclophosphamide, streptonigrin and prednisone. These agents resulted in stabilization of the lymphocyte count, but did not correct either the thrombocytopenia nor the adenopathy. In August 1974, the patient had a rapidly increasing lymphocyte count, massive adenopathy in the neck, chronic low grade fever, progressive weight loss and other signs of hypermetabolism. He was treated with total nodal radiotherapy, approximately 2000 rad to all regions, but while this resulted in regression of the node masses in the neck, there was little effect on other symptoms and signs of the disease.

In December 1974, an arteriovenous fistula was created in the right arm. The patient was then leukapheresed using the IBM–NCI cell separator a total of 18 times in a 30-day period, removing a total of 5.6×10^{12} cells. At the beginning of leukapheresis, the patient's peripheral white blood cell count was 210,000. There was a rapid drop in his counts to approximately 10,000. During the ensuing 3 months, the patient maintained his absolute blood lymphocyte count between 15–25,000/mm^3, showed regression of neck adenopathy, a loss of fever, a gain in weight and a partial correction of both thrombocytopenia and anemia. Bone marrow samples obtained during this period showed gradual increase in myeloid and erythropoiesis with reduction of lymphocytosis. One further leukapheresis procedure was done in late March for biochemical studies. Because the disease appeared stable and the patient seemed to be in better clinical condition than he had been in the previous 2 yr, no further therapy was given.

*Stage O, bone marrow and blood lymphocytosis only; Stage I, lymphocytosis with enlarged nodes; Stage II, lymphocytosis with enlarged spleen or liver or both; Stage III, lymphocytosis with anemia; Stage IV, with thrombocytopenia [3].

RESULTS

Ribonucleotide profiles

The ribonucleotide profiles of cells obtained at different stages of the disease in patient PM are shown in Fig. 1. Figure 1(a) is a typical HPLC chromatogram showing the complete ribonucleotide contents of CLL cells and is representative of profiles obtained on 5 different occasions between April 1974 and May 1975.

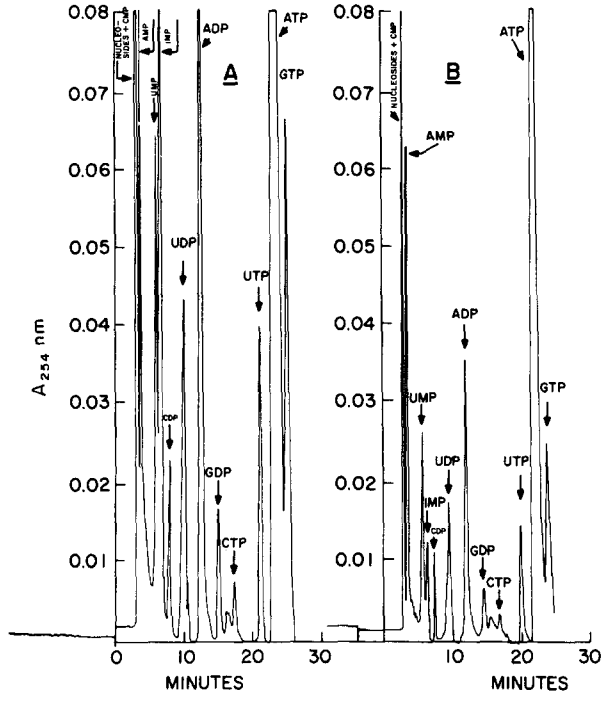

Fig. 1. High pressure liquid chromatography of acid soluble extracts of cells obtained from patient (PM) with chronic lymphocytic leukemia. A represents a typical chromatogram of cell extracts of the patient before "transformation" (6/74–6/75) and B after "transformation" (7/75–11/75). Cells (1.0×10^8) were extracted with 1.0 ml of 6% PCA and the supernatant was then neutralized to pH 7.0 with 2N KOH. Ten microliters (about 10^6 cells) of such an extract was analyzed on an HPLC (see Methods).

Table 1. *Characteristics and previous treatments of patients*

			At diagnosis		At testing					
Patient	Age	Duration from diagnosis to 1st study (months)	Stage of disease	Lymphocyte count $\times 10^3$	Stage of disease	Lymphocyte count $\times 10^3$	Date	Current Rx	Previous Rx	Other remarks
ML	73	5	I	28	III	126	4/7/75	Chlorambucil	0	0
						20	11/17/75	Prednisone		
JA	70	24	II	25	III	23	2/23/76	Vincristine	Chlorambucil	
							3/10/76	Cytoxan	Prednisone	
VL	71	28	III	170	IV	1300	11/19/75	Cytoxan	Chlorambucil	Uremia,
						259	12/2/75		Prednisone	hypouricemia 11/19/75
GF	66	6	IV	645	IV	49	10/27/75	Vincristine	Chlorambucil	Uremia,
						28	12/22/75			hypouricemia, congestive heart failure at diagnosis
CB	58	4	IV	239	IV	790	11/21/75	Chlorambucil	0	0
						175	1/8/76	Prednisone		
PM	55	144	II	70	IV	364	4/12/74	See case	Chlorambucil	Pulmonary
						136	6/6/74	report	Prednisone	infection
						58	1/12/75		Tetramethyl-	(viral)
						21	5/5/75		melamine	1/12/75
						180	7/20/75		Cytoxan	Hypouricemia
						86	7/30/75		Streptonigrin	
									Radiation therapy	Disease

Striking features are in the area of the monophosphates, especially IMP, and in the area of the triphosphates, in particular ATP. The ratio of ATP to IMP in Fig. 1(a) is about two. Similar findings were observed when the acid soluble fraction of the cells was analyzed using a DEAE column chromatographic method [7]. Figure 1(b) shows the ribonucleotide profiles of cells obtained from the same patient in June 1975. This chromatogram differs from that shown in Fig. 1(a) in that the pools of IMP are sharply reduced with concomitant increase in the pools of triphosphate, especially those of ATP and GTP. Progressive increase in the ribonucleotide triphosphates were observed with time, reaching a maximum in August 1975 (data not shown).

The ribonucleotide profile of the cells obtained from the other 5 patients were also analyzed and the data are summarized in Table 2. In one patient (JA) a ribonucleotide profile, reproducible on 2 occasions, similar to that shown in Fig. 1(a) was obtained. At a later date, however, (February 1975) the IMP peak was reduced drastically with a concomitant increase in the ATP pool. These biochemical changes in the ribonucleotide profile were observed simultaneously with the ability of cells to utilize glycine and thymidine (see below). Thus, in 2 of 6 patients (PM and JA, Table 2) a shift from a low ATP/IMP ratio (1.4 and 1.3 respectively) to a higher ratio (14 and 30 respectively) was observed. In the other 4 patients, the profiles consistently resembled that of Fig. 1(b).

In vitro *precursor activation and incorporation into nucleic acids*

The uptake of glycine and orotic acid into cells and their incorporation into ATP and UTP, respectively, was measured as an indicator for determining the ability of cells from patients with CLL to utilize *de novo* purine and pyrimidine synthetic pathways. Incorporation of AR into ATP and of TdR into nucleic acids was measured as a parameter for evaluating the ability of CLL cells to utilize salvage pathways of purines and pyrimidines, respectively.

The ability of CLL cells to utilize salvage and *de novo* metabolites were further investigated in the other five patients. The data are summarized in Table 3. In one of these five patients (JA) a significant and reproducible increase in glycine utilization was observed at later stages. The data also show that the cells obtained from the 2 patients (PM and JA), at their initial presentation, possessed a lower profile in their ability to utilize glycine and TdR than these cells obtained from the other 4 patients. In contrast, GF cells utilized salvage and *de novo* metabolites relatively better than cells obtained from the 5 other patients. These cells were characterized as typical T type while the cells of the 5 other patients were typical B type (J. Minowada, Personal Communication).

The results of the studies carried out on the cells from one CLL patient (PM) at various stages of the disease are shown in Fig. 2. In the early stage of the disease (July 1974–May 1975) when the patient was clinically stable and for several months thereafter, these cells possessed a limited capacity to use glycine and thymidine for nucleic acid synthesis. The incorporation of AR was, however, rapid. From July onward the ability of these cells to utilize glycine and TdR increased progressively while the incorporation of AR into nucleic acids was not significantly altered. These biochemical changes preceded

Table 2. Ribonucleotide pool sizes in patients with chronic lymphocytic leukemia

		μmole/g of cells*					
Patient	Stage	IMP	CTP	UTP	ATP	GTP	ATP/IMP
GF	IV	0.2±0.11	0.18	0.22	2.9±0.17	0.42	14.5
PM	IV	0.86±0.28†	0.09	0.15	1.2±0.38	0.20	1.4
		0.25±0.12‡	0.26	0.42	3.6±0.46	0.68	14.4
ML	III	0.17	0.08	0.24	1.4±0.22	0.28	8.3
VL	IV	0.23±0.08	0.06	0.13	1.8±0.30	0.24	7.8
JA	III	0.62±0.33†	0.64	0.08	0.83±0.21	0.1	1.3
		0.08‡	—	0.16	2.4±0.11	0.42	30.0
CB	IV	0.18	0.11	0.42	2.8±0.62	0.5	15.5

*Except for PM and TA, these values represent an average of 4–8 separate analyses with 1–2 month interval between each analysis.

†During the period of July 1974–May 1975 (average of 9 analyses with a value ranging from 0.42–1.18). *P* value significant <0.001.

‡During the period of August 1975–October 1975 (average of 6 analyses).

Table 3. Utilization of de novo *and salvage metabolites by CLL cells*

	Counts/min/10^7 cells*			$\mu g/10^7$ cells	
Patient	Glycine	AR	TdR	DNA	RNA
ML	394 ± 28	6280 ± 726	570 ± 120	70.3 ± 11.9	26.0 ± 8.4
GF	4380 ± 322	10842 ± 2012	726 ± 56	—	—
VL	572 ± 78	6800 ± 898	874 ± 78	27.2 ± 3.5	4.2 ± 1.5
CB	501 ± 101	4900 ± 286	896 ± 102	32.8 ± 6.8	14.8 ± 21
JA	175 ± 42†	8900 ± 1320	105 ± 50	43.8 ± 5.3	18.9 ± 4.8
	1064 ± 112‡	7850 ± 632	632 ± 120	62.4	26.8
PM	40 +	4200 ± 1060	20	25.0 ± 5.6	10.3 ± 3.8
	2808 ± 421‡	4400 ± 2280	832 ± 126	62.8 ± 4.7	21.4 ± 6.8

Cells were incubated with the labeled metabolite for 30 min. Acid-soluble fractions were then analyzed by HPLC for the amount of label found in ATP when glycine and AR were the precursors under investigation. The amount of radioactivity incorporated into DNA was determined when TdR was used.

*These studies were always carried out in duplicate and the values obtained were within 10% of each other. ± Refers to the standard deviation of the values obtained from multiple observations on the same individual at different times. The number of analyses in each patient varied from 4–8 analyses each separated by 1–2 months.

†Before leukapheresis.

‡After "transformation" of disease. See text.

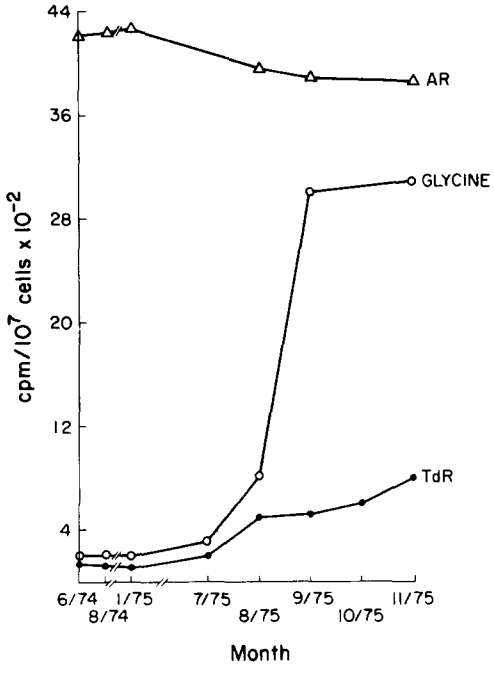

Fig. 2. Ability of cells obtained from a patient with CLL (PM) to utilize a de novo *(glycine) and salvage metabolites (TdR and AR) at various times during disease progression.*

any distinct change in the clinical status of the patient. At the time of the appearance of a large tumor mass in the right groin, tissue biopsy and bone marrow cells were also analyzed for their ribonucleotide profile and for the ability of the cells to utilize *de novo* and salvage metabolites. The material obtained from the solid tumor mass utilized glycine and thymidine considerably more efficiently than cells obtained from peripheral blood. In addition, the ribonucleotide triphosphate pools of cells obtained from the solid mass were over two fold greater than those found in cells obtained from the peripheral blood of the same patient (data not shown).

Relationship between nucleotide pool ratios and tumor cell reliance on de novo *and salvage pathways*

Differences in the ability of L-1210, P-288, Taper hepatoma and CLL cells to utilize *de novo* and salvage metabolites for nucleic acid synthesis are shown in Table 4. The data indicate that there is a direct relationship between the ATP/IMP ratios and the ability of cells to utilize *de novo* metabolites. The larger the ratio (e.g., Taper hepatoma), the greater the dependency of these cells to utilize metabolites for *de novo* nucleic acid synthesis. Conversely, the smaller the ratio (CLL cells) the greater the dependence of these cells on salvage pathways. The data in Table 4 also show that 6-MP, a known inhibitor of *de novo* pathways of purine biosynthesis produced a significant increase in the life span of mice bearing Taper hepatoma, while it produced only a marginal increase in the life span of mice bearing L-1210 cells.

In vitro *metabolism of selected antimetabolites*

Cells obtained from the peripheral blood of patients with CLL were incubated *in vitro* with ara-C-^{3}H. Thirty minutes thereafter, the acid soluble fractions were prepared and analyzed for their content of labeled ara-C and its known anabolic and catabolic products. Results of these studies are summarized in Table 5. The results

Table 4. *Relationship between reliance of tumor cells on salvage and* de novo *pathways for nucleic acid synthesis and drug sensitivity**

	Salvage†	*De novo*	ATP/IMP	Increase in survivors‡ 6-MP (%)
L-1210	+ + + +	+ +	2	25 (0/10)
Taper Hepatoma	+ + +	+ + + + +	20	80 (4/10)
P-288	+ +	+ + +	2	N.D.
CLL	+ + + +	—	1.1§	N.D.

*For evaluation of the ability of tumor cells to utilize *de novo* (glycine-8-^{14}C) and salvage metabolites (AR-8-^{14}C), 5.0×10^7 cells were incubated at 37°C for 30 min. Acid-soluble fraction was analyzed for labeled ATP and acid-insoluble for incorporation into nucleic acids.

† + + + Means predominantly dependent on salvage pathways for nucleic acid synthesis, + + + less dependent on salvage pathway but more dependent on *de novo* pathways.

‡Mice bearing the designated tumors were treated i.p. with 6-MP (125 mg/kg × 1) 24 hr after inoculation of 10^6 tumor cells. The average survival time of control mice bearing the L-1210, Taper hepatoma and P-288 were 7.8, 19.8 and 12.6 days respectively. Parentheses indicate the number of mice which survived 60 days over the total number of mice.

§Two out of the six patients with CLL.

Table 5. In vitro *metabolism of ARA-C-5^3-H in CLL cells*

	Counts/min/10^7 cells			
Patient	ara-CMP	ara-UMP	ara-CDP	ara-CTP
GF	2862	976	1062	28,622
PM	1033†	1982	733	38,722
ML	3095	3862	353	40,131
VL	517	362	—	11,517
JA	3622	8622	735	7562
CB	5866	14,622	826	5322

Cells were incubated at 37°C for 30 min with the labeled drug. Acid-soluble extracts were then analyzed for ara-C metabolites using an HPLC system.*

*In all the patients tested, the amount of intracellular ara-C was 200–600 and ara-U 175–465 counts/min/10^7 cells.

†After "transformation" of the disease.

indicate that these cells transport and activate ara-C to the triphosphate level. The amount of ara-CTP formed after 30 min incubation with ara-C varied from patient to patient. Furthermore, the amount of ara-UMP formed was found to be high in two patients (JA and CB) while in the others it was quite low.

Clinical diagnosis and treatment

On 29 July, 1975 the patient (PM) presented with no changes in his hematologic parameters but with a sensation of weakness and the appearance of a large node in the right groin. Aspiration of this node was consistent with CLL. A marrow sample obtained at this time showed that the percentage of lymphoblasts had increased from 6 to 12%. Biochemical measurements showed that the DNA and RNA content per cell had doubled and the average cell size of lymphocytes (measured by ocular micrometry) was nearly doubled.

The patient was treated with 3 hr infusions of 6-MP, 600 mg/m^2 i.v. daily for 5 days. At the beginning of treatment the patient's total lymphocyte count was 224,000 and the patient had a serum calcium of 12.5 mg %. The treatment did not result in a significant change in the calcium level nor in the size or firmness of the lymph nodes in the neck but the white count gradually dropped to an absolute lymphocyte count of 85,000 with marked thrombocytopenia (7500/ml). Upon recovery of the platelets to pretreatment levels of 65,000/ml the patient was placed on ara-C 100 mg/m^2 by continuous daily infusion for 5 days. This treatment resulted in a drop of the total white count from 99,000 to 70,000 in a 7 day period. In addition, the calcium dropped from 12.5 to 9.5 mg % and the

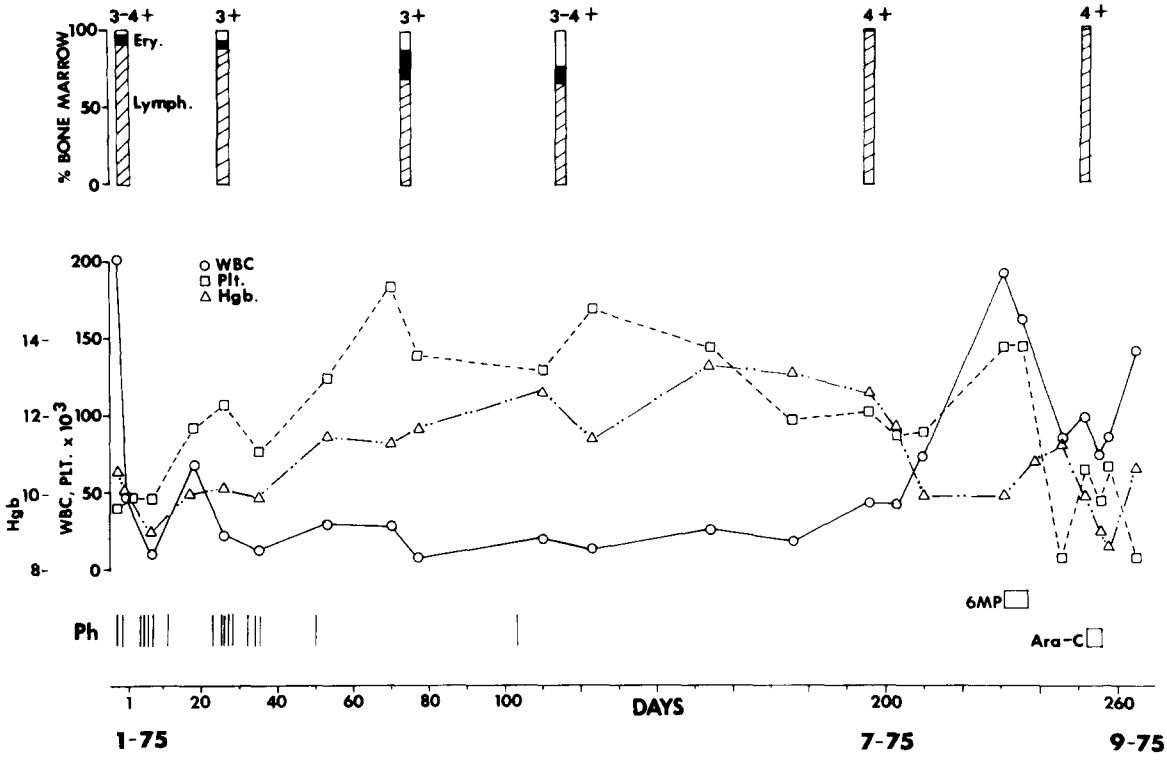

Fig. 3. Clinical course following remission induction with leukapheresis (PM). Biochemical and clinical changes in the character of the disease were noted at day 200. *6-Mercaptopurine and cytosine arabinoside were given as indicated (see text).*

lymph nodes regressed in volume by 25% with marked softening. The patient's appetite improved although low grade fever continued to be present. Seven days layer, the patient developed marked absolute granulocytopenia together with pneumonia and septicemia and expired, this being 13 yr after diagnosis. Figure 3 is a summary of this patient's course from the time of leukapheresis treatment through the period of transformation and the treatment with 6-MP and ara-C.

DISCUSSION

Chronic lymphocytic leukemia (CLL) is a disease in which the abnormal lymphocytes resemble their normal counterparts morphologically, but show significant alterations of functional integrity. The disease is characterized by a variable course, ranging from a benign lymphocytosis to an aggressive disease characterized by ever-increasing numbers of cells, infiltration of hematopoietic lymphoid and other organs, associated defects in immune system, defects in the production of normal marrow elements, hemolytic anemia and susceptibility to severe viral infections. Therapy may not be necessary in the early stages, while in the more advanced phases control is often achieved with alkylating agents and prednisone. As the disease progresses, however, current therapeutic modalities are usually unsatisfactory and disease-related death ultimately occurs.

The management of CLL, chronic myeloblastic leukemia and so-called "prolymphocytic leukemia," with intensive leukapheresis has been described [8–10]. This study documents the temporary effectiveness of this mode of therapy in one of the patients studied. Following short-term intensive leukapheresis, the patient showed progressive improvement in all the parameters of his disease, including repopulation of the marrow with normal elements. Leukapheresis may have altered the characteristics of the disease more fundamentally, perhaps by creating a more favorable ratio between normal marrow stem cell elements to the leukemic precursors. Whether the terminal "aggressive" phase of the disease in this patient may have been influenced by leukapheresis is not known. The experience of others would suggest that this type of intervention is at least not harmful.

Attempts have been made to characterize the disease by *in vitro* phytohemagglutinin (PHA) stimulation [11], chromosomal markers [12, 13] and serum protein levels [7]. More recently, Scholar and Calabresi [4] have shown that some CLL cases are characterized by the inability of

the leukemic cells to utilize glycine as a *de novo* precursor for nucleic acid synthesis. These cells thus appear to rely predominantly on salvage pathways for maintaining cell function.

In this report, attempts were made to characterize biochemically CLL patients at various clinical stages of their diseases. These studies included the quantitative estimation of ribonucleotide pools in particular, the ratios of ATP/IMP, the ability of the cells to utilize *de novo* vs salvage metabolites, and the measurement of the extent of metabolic activation of selected antimetabolites such as ara-C. These studies were carried out simultaneously on cells obtained from each patient at various visits to the hospital. Such studies might be of value in (a) the establishment of baselines for the biochemical profiles of CLL cells during early ("benign") stages of the disease; (b) determining whether or not changes in the biochemical profiles can be used to predict impending clinical changes in the disease; and (c) evaluation of the use of these tests to predict the sensitivity of CLL cells to selected antineoplastic agents. This report is a preliminary study summarizing the results of such investigations in 6 patients.

The data reported here confirms the studies of Scholar and Calabresi [4]. Cells from 6 patients with CLL were shown to possess a reduced ability to utilize purine and pyrimidine *de novo* metabolites, as indicated by the lack of formation of ATP and UTP formation from glycine and orotic acid respectively. However, in one of our patients (PM) although the CLL cells initially lacked the ability of utilizing glycine and orotate, TdR and glycine incorporation into nucleic acid progressively increased, preceding the accelerated phase of his disease. This increase reached a plateau within 2 months prior to death. In another patient, JA, the ability of cells to utilize glycine for the formation of ATP developed coincidentally with the occurrence of advanced and aggressive disease. Coincident with these shifts from a major reliance of cells on salvage pathways for nucleic acid synthesis, to at least partial dependence on *de novo* pathways, a corresponding change in the ribonucleotide profile was observed. Table 2 illustrates the shift from lower to higher ATP/IMP ratios. Prior to the shifts, repeated observations on different occasions had remained relatively constant.

However, in the 4 other CLL patients, there was no correlation between high ATP/IMP ratios and the ability of these cells to utilize *de novo* metabolites. Although nucleoside triphosphate pools in the cells of these latter patients were relatively high, their CLL cells maintained predominantly salvage pathways for providing precursors for nucleic acid synthesis.

It is thus conceivable that the dependence on salvage pathways represents a homeostatic mechanism for the control of proliferation of CLL cells, corresponding to the rather indolent "accumulative" phase of the disease, and that more aggressive disease may be a consequence of the development of clones of cells with capabilities for *de novo* synthesis of DNA. This hypothesis remains to be tested.

In 3 animal tumor cell lines the antitumor activity of 6-MP seems to correlate with the ability of these cells to utilize salvage and *de novo* (glycine) metabolites. The uptake of AR in CLL was of the order of magnitude of Taper hepatoma and P-288. In contrast, the uptake of a *de novo* metabolite was found to be lowest in L-1210, next in P-288, and the greatest in Taper hepatoma. In these animal tumor systems, the ATP/IMP ratio was inversely correlated with the ability of these cells to utilize salvage metabolites. In tumors with an active *de novo* pathway, antimetabolites capable of inhibiting these pathways may be most effective. Indeed, the results show that treatment of mice bearing Taper hepatoma with 6-MP produced long term survival. In contrast, in L-1210, a tumor with a relatively marked dependence on salvage pathways, 6-MP produced only a 25% increase in survival time.

In summary, although the results of this investigation do not allow us to draw a definite conclusion at this time, they point out the need for determining in more patients whether or not fluctuation in the ribonucleotide pools and their ratio and changes in the balance between *de novo* and salvage pathways may be used as an early warning sign for an impending change in the clinical status of CLL patients. If it turns out that the changes in the ATP/IMP ratios and the dependence on salvage vs *de novo* pathways do not correlate with impending clinical crisis in every CLL patient, in those patients with positive correlation measurement of these parameters may have some value in the early management of the diseases with chemotherapy.

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Non-African Sporadic Burkitt's Lymphoma in Italian Patients

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Abstract—*A peculiar tumour of children, similar for certain histological and biological features to Burkitt's lymphoma has been recognized in Italy, probably a B lymphoma.*

The serological data are not consistent with the same aetiology of African cases, except for one of 8 patients (5 tested appropriately). "Maxicure with minitreatment" after cyclophosphamide has been reported for African cases; similar results have been achieved in 3/8 patients of this series with the adriamycin + vincristine combination; this combination probably deserves larger testing also in African cases. Four out of eight patients are faring well, at the time of reporting.

INTRODUCTION

THE PECULIAR lymphoma recognized in African children by Burkitt§ was earlier regarded as a nosological entity confined to certain geographical areas. Later on, a similar condition began to be recognized in Europe and the States [1, 2].

Pathological (and cytological) features of these 2 clinical entities are identical [3] while the serology and pattern of distribution of the tumour deposits seem to be at variance from country to country [1–4]. Furthermore only occasionally EBV (Epstein–Barr virus) infection can be found responsible or correlated in cases described outside Africa.

In the last years, we have observed 8 patients with immunological, morphological and clinical features resembling, more or less, the African cases. The possible leukemic evolution and the low titres of EBV antibodies, however, suggest the existence of an autonomous form of the disease in Europe or, at least, in Italy.

MATERIAL AND METHODS

The age of our patients varies between 3 and 19 yr; illness duration at admission had been very short (4–8 weeks). Regarding *presentation*, 1 girl at the admission was already leukemic‖; 1 boy progressed in 4 months to a leukemic picture; 4/8 patients, however, were thrombocytopenic and anemic since the beginning; Table 1 shows an outline of epidemiological and presentation data. Tumours were clinically staged as follows: stage I—localized facial tumour; stage II—two or more facial tumours; stage III—intrathoracic, intra-abdominal, or osseous tumour (excluding jaws) and paraplegia; stage IV—meningeal or generalized bone marrow metastases, according to I. T. Magrath (1975).

Two patients had a maxillary tumour, and 2 paraplegia from spinal involvement. Two patients presented with fever and weight loss (systemic signs) from the start.

Histopathology

Paraffin sections after formol fixation and hematoxylin + eosine stain have been the basic diagnostic tool.

Cytological studies

Bone marrow aspirates and imprints from biopsy specimens have been obtained for all patients. All these preparations were stained

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§African lymphoma; Burkitt's tumour are synonyms; for cases described outside the endemic area, Burkitt-like tumour has been the commonly adopted eponym.

‖She was earlier admitted to another hospital and it is difficult from reported data to know if she was leukemic on the first admission.

Table 1. General features of the patients with Burkitt-like lymphomas (status at presentation)

Identification				Presentation										Serology*					
No.	Age	Sex	First Sign	Peryph. Blood	Bone marrow	Superf. nodes	Mediast.	Lung	Pleur.	Bone	Para-plegia	Jaw maxil.	Abdom.	Stage	EA	VCA	EBNA	Surface IG	Social class	Living area*
Patient																				
1	11	M	Cough	(late)	(late)	late	+	+	+					III°					L	NIC.
2	4	M	Dry throat			+ and tonsils			(late)					III°					L	NIC.
3	6	F	Para-plegia	+	+						early	(late)		IV°	10	32	160		M	NIT.
4	19	F	VII°N. palsie			+				+		+		IV°	10	20	160	Monoclonal IgM	M	NIT.
5	6	F	Nodes			+	+	+		+	early			IV°	10	320	—		L	NIC.
6	10	M	Sinus-itis				+	+				+		III°	10	80	32	Monoclonal IgM	M	SIT.
7	14	M	Sinus-itis	+	(late)	+	+		+					III°		Lost sample			M	NIT.
8	3	M	Invag-ination										+	III°	80	320	320		L	NIC.

EA = early antigen—VCA = virus capside antigen—EBNA = Epstein–Barr nuclear antigen.
*Under 'living area' heading – NIC means Northern Italy, country; SIT means Southern Italy, town.
In 4/5 patients the serology was retested when in remission without any change in EA, VCA, EBNA titre.

according to the May Grünwald Giemsa method. Node aspirates were performed when possible. In some patients these studies have been repeated on spinal fluid and other specimens (e.g. pleural fluid).

For cytochemistry, Methyl Green Pyronine, P.A.S. peroxidase and Oil Red O have been also used. Cell markers have been detected by *immunological methods* on marrow or biopsy specimens using Hyland antisera tagged with fluorescein and monospecific anti gamma, alpha, mu, K and lambda activity [6]. Cell suspensions were observed after this preparation through an Orthoplan Leitz microscope for immunofluorescence. Jondal method [7] has been used to study spontaneous rosette formation with sheep erithrocytes.

Serology and virology

Studies for early antigen; membrane antigen and viral capside antigen and for Epstein–Barr nuclear antigen (EA; MA; VCR; EBNA) have been made at presentation and in several other occasions according to the standard techniques [8–10]. In the last patient nuclear hybridization studies for EBV components in the tumour cell genoma has also been performed [11].*

RESULTS

Epidemiology

Previous infectious mononucleosis was not reported in our patients on careful questioning; one patient came from an area of endemic malaria; social status varied from low to middle class (manual worker to school teacher); geographic distribution was haphazard from all Italy; no clustering of tumours of the lymphocytic system or tumours affecting young patients was discovered on enquiry.

Pathology

A starry sky appearance with large lymphocytic sheets and scattered non-malignant macrophages was the common feature of 8/8 patients (Figs. 1 and 2).

Cytomorphology

The blast-like cells found in the tumour imprint in some patients invaded the circulation and bone marrow; they were poorly differentiated with a moderately expanded basophylic cytoplasm showing many vacuoles of 1–2μ dia. Rounded or slightly oval nuclei with a delicate chromatine net-work and one prominent nucleolum were usually seen (Fig. 2). MGP stain as well as Oil Red O were positive in the cytoplasms. Negative reactions from peroxidase and PAS stains were obtained in all specimens.

Lymphocyte markers

Only two cases, 4 and 7, have been studied for surface markers (1) of the malignant cells; both had on the cell membrane distinctive spots of a monoclonal surface marker (Sm. Ig.) of IgM type with lambda light chains. Fluorescence was relatively strong. No spontaneous rosette formation was observed.

Serological studies

Table 1 shows that only two patients had titres similar to African lymphoma; one with only VCA and one with both VCA and EBNA. These patients had not suffered from infectious mononucleosis and gave a negative Paul Bunnel reaction. An attempt to molecular hybridization gave negative results in one patient (serologically negative) and no study of this type has been performed on the other patients.

Response to treatment

The first patient was treated with a loading dose of cyclophosphamide with only transient improvement. Then radiotherapy to bulky disease and combined chemotherapy as for acute lymphoid leukemia (ALL) proved slightly effective; this boy died after a 12 month course. One patient failed to respond to the ABVD scheme [12] and is now in full remission at 20 months after treatment according to the L10 protocol [13]. Four patients began the treatment with one 24 hr course of adriamycin (65 mg/m^2) followed at 48 hr by vincristine 1 mg/m^2; 3 of these patients are now living and well at 18, 36 and 54 months (one of them received further adriamycin as maintenance treatment for 6 months). The fourth of these 4 patients, despite adriamycin maintenance relapsed and died at 3 months. Adriamycin + vincristine, according to the above-mentioned schedule, was also applied as second treatment of choice after radiation failure in a 5th patient, who nevertheless died at 12 months. Of 3 patients treated for relapse according to the L10 scheme, 1, No. 7 in Table 1, is now free of evidence of disease at 20 months, and 2 died (1 at 44 and 1 at 12 months from the beginning). Table 2 summarizes these data.

*Sera and tumour specimens for these studies have been mailed with dry ice in RPMI with 20% FCS and antibiotics to the Lyon International Cancer Agency, France. We acknowledge here the friendly cooperation of Dr. G. Blaubin De Thé to this work and of Dr. N. Munoz for revision of the slides.

Table 2. Clinical course with respect to treatment

Patient no.	INIT. TREAT. ADR.	VCR.	Cyclo-phosph.	Pred.	Other	Subseq. treat.	REMISSION P.R.	C.R.	Site	RELAPSE Delay (mo.)	Other sites	Last follow-up (Dead)	N.E.D.	Length surv. off Treat. (mo.)
1		+	+	+		6MP MTX	+		Scalp	3	Blood, marrow, lung	12		—
2	+	+			Mantle rad	Pred. VCR		+	Kidney	3	Pleura	6		—
3	+	+			rad	L10		+	Bones	12	Jaw	26		—
4	+	+	+	+	rad		+		Palate	1	C.N.S.	2		—
5	+	+				—		+	—	—	—		60	60
6	+	+				ADR		+	—	—	—		22	18
7					ABVD	L10	+	+	B.M.*	1	Nodes		10	4
8	+	+					(Post-oper.)	+	—	—	—		36	34

Init. treat. = describes the single or combined treatment given to obtain the first remission.
ADR = adriamycin; VCR = vincristine; cyclophosphamide; Pred = Prednisone.
ABVD see text and references (Bonadonna).
Subseq. treat. = maintenance and/or second choice treatment given respectively, to remitters or to non-remitters after init. treat.
P.R. = partial remission

C.R. = complete remission
mo. = months.
} according to E.O.R.T.C. criteria.

N.E.D. = no evidence of disease.
Length surv. off. treat. = survival after stopping all anticancer treatment.
*B.M. = bone marrow.

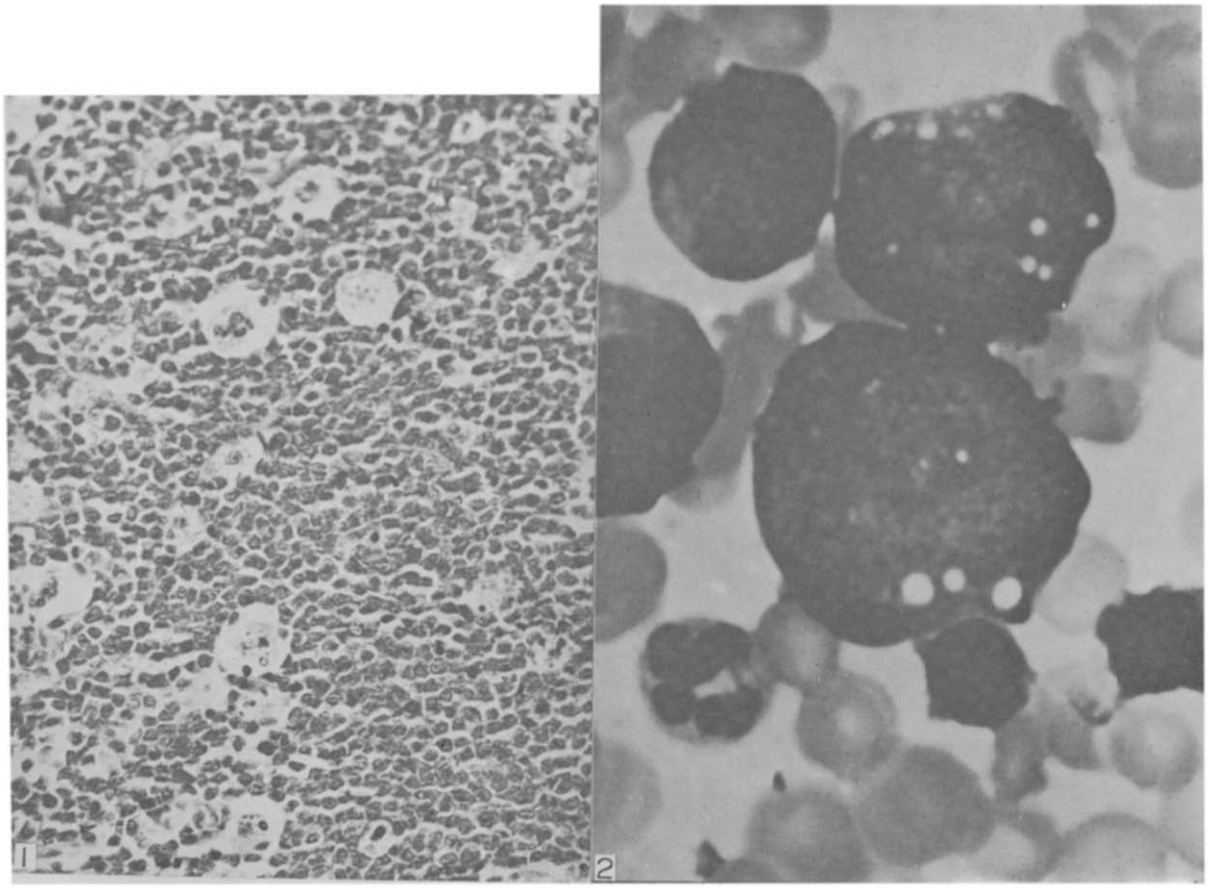

Fig. 1. *Typical hystologic picture of case* No. 1. *Em.Eo. magnification* × 360.

Fig. 2. *Citology of case* 3. *In this case the characteristics of the cells were clear, from a qualitative point of view. At variance was the exaggerated magnitude of some of the lymphoblastic cells.*

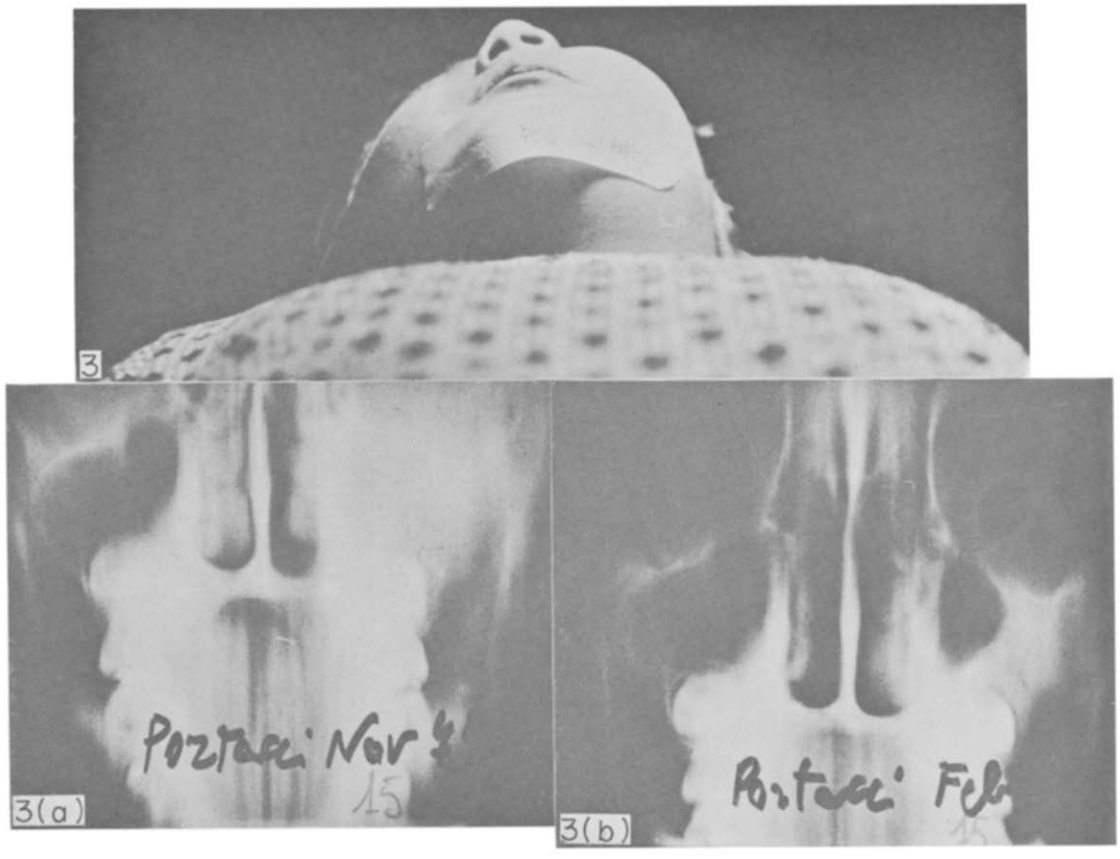

Fig. 3. (a, b). *An axial view shows the prominence of the upper maxillary bone in case* 6.3. *The laminagram at* 15 cm *from the dorsal plane shows involvement of bone and sinus cavity* (3a) *at admission, while a film taken* 4 months *later with the same technique* (3b) *shows a partial remission. The syncronic disappearance of mediastinal and lung involvement suggested discontinuation of adriamycin from March. The patient is symptom free at* 14 months *from treatment discontinuation.*

DISCUSSION

In 6 yr, 8 patients (among ~ 10,000 admissions) suggested a Burkitt-like diagnosis to our pathologist (N.P.) on paraffin sections and on cytological examination. Four out of eight patients are now in full unmaintained remission, 3 of them after a brief course of adriamycin + vincristine.

The bone involvement, often initially observed, appeared earlier or later in 7/8 patients and should be considered a prominent feature. The tumour cells appeared to behave as monoclonal B cells [1] in the 2 cases subjected to full immunocytological studies, in good agreement also with Binder [14] and Epstein's data [15] for sporadic cases of non-African Burkitt-like lymphoma. Tumour sites included twice the maxillary bone (lower incidence than in true BL (Fig. 3), and twice also the central nervous system + lymph nodes (higher incidence).

Two out of eight cases became frankly leukemic (1 of them had a leukemic picture since the beginning), while in Africa the bone marrow infiltration usually appears unaccompanied by peripheral blood invasion [16]. This feature however may be prominent [3] in American cases (50%) and has been repeatedly reported in Europe and in the United States of America [1, 17].

Table 3. Clinical evolution in dying patients

Death modality	Patients (No./total)	(%)
Leukemic transformation	2/8	25
Progression of tumour masses	2/8	25

The serology of only 1 patient was in full agreement with the African cases; the prominent features of this case were abdominal localization and unmaintained remission at 3 yr after ileal resection and a short course of adriamycin + vincristine. The cyclophosphamide "maxicure with minitreatment" policy, sometimes effective for African cases, has been tried only once and with a poor outcome in our patient, No. 1, while it has proved successful with adriamycin + vincristine in cases 5, 6 and 8.

A good outcome seems possible (although not warranted for the moment) also in case 7, treated as ALL with the L10 protocol followed by radiotherapy on the mediastinum and is now off treatment since 4 months [13]. Radiation treatment and prolonged multiple chemotherapy have not proved to be successful in 4 patients.

Our feeling is that the cases described pertain to a separate entity among the B lymphomas, non-Hodgkin type, of children. This entity has pathology, pathogenesis, clinical behaviour, tumour-host relationships similar to African lymphoma, but is probably related to aethiological or triggering factors different from EB virus and, possibly, different from patient to patient.

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Tumor Growth Inhibition Mediated by Trypsin Inhibitor or Urokinase Inhibitors*

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Abstract—*When testing* in vitro, *we often observed that erythrocytes taken from Ehrlich ascites tumor bearing mice displayed enhanced agglutinability by the lectin Concanavalin A, suggesting that protease activity operates* in vivo.

In several studies, we were able to inhibit Ehrlich ascites tumor growth by the repeated administration of soya bean trypsin inhibitor. Studies of dosages and schedules of treatment showed that for 20,000 *initially grafted cells, treatment resulted in* 0–70% *of long-term survivors and induced a significant increase in mean survival time of treated mice over control mice. For* 200,000 *grafted tumor cells,* 0–40% *of long-term survivors were recorded.*

In a comparative study, we found that different inhibitors of urokinase displayed a similar chemotherapeutic effect against 200,000 *inoculated cells. Our results corroborate the idea that a plasminogen activator monitored chain of fibrinolytic and proteolytic activity controls tumor growth and metastasis enzymatically.*

INTRODUCTION

THERE is growing evidence that proteolytic and fibrinolytic enzymes are involved in tumor growth and metastasis: *in vitro* studies of cell line cultures have shown that normal cells grow to monolayer cultures, reach reproducible cell saturation densities and obey growth inhibition by cell–cell contact [1]. Resting cells can be triggered into mitosis if some hormones (insulin), lectins (Concanavalin A) or protease (trypsin) are added to the cultures [2]. Tumor cells, however, pile up each other, attain higher cell saturation densities and die if they are not transferred to fresh culture medium [3].

So, it was proposed that tumor cells, unlike normal cells, carry or secrete their own mitotic protease [4]. Since 1915 it is known that mammalian tumor cells lyse clotted plasma substrates [5]. Whereas normal fibroblasts and cultures of normal tissues release little if any fibrinolytic enzymes, it was reported that mammalian fibroblasts transformed by DNA or RNA viruses, chemically or virally induced carcinomas and human malignant tumors in culture release substantial quantities of fibrinolytic enzymes [6]. Reich *et al.* clearly demonstrated that this fibrinolytic activity is generated by the interaction of serum plasminogen [7] with a cellular activator of plasminogen (PA) found in the supernatants of tumor cell cultures [8]. Further studies revealed that PA is also associated with untransformed cells, such as macrophages [9] and human fibroblasts [10] and that PA is to be induced in those cells.

Very likely, this PA, after its induction by the coagulation enzymes and inflammatory agents [11], may physiologically monitor the migration (such as macrophage diapedesis) of those particular cell types active in wound healing processes and enable those cells to transect the structural barriers encountered during migration [12]. Tumor cells continuously produce PA and are capable of migrating in wounds [13]. Furthermore, it is clearly established now that patients with advanced cancer and lymphoid malignancies often show impaired cellular and humoral immune responses [14]. Micromolecular circulating fibrin and degradation products of plasminolytic activity, have shown to induce a

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time-dependent and dose-dependent immunosuppressive action in the early phase of clonal proliferation of immunocompetent cells *in vitro* and *in vivo* [15] and were claimed to be responsible for immunosuppression. PA activity was found to be associated with the plasma membrane in Rous sarcoma virus transformed chick embryo fibroblasts [16] and Funahara *et al.* demonstrated the presence of PA in extracts of Ehrlich ascites tumor cells [17].

Recently, Astedt and Holmberg were able to demonstrate an immunological identity of urokinase and ovarian human carcinoma PA released in tissue culture [18]. Possibly, this enzyme, after its secretion by the tumor cell, may activate serum plasminogen and initiate a chain of proteolytic activities on neighbouring cells, tissues or serum proteins in the microenvironment of the tumor cell. In this study, we investigated if the administration of trypsin inhibitor of soya bean or urokinase inhibitors could influence the growth of murine ascites tumors.

Soya bean trypsin inhibitor is the naturally occurring macromolecular inhibitor of trypsin, plasmin, kallikreins, chymotrypsin and prothrombin convertase [19]. Urokinase is a 31,000 or 54,700 dalton molecule with proteolytic specificity for plasminogen [210].

Enzymatic activity can be inhibited by inhibitors of serine proteases such as para-amino-benzamidine and epsilon-amino-*n*-caproic acid [21].

MATERIALS AND METHODS

Material

Concanavalin A was a 10% protein powder obtained from Sigma (lot No. 94C5015) with specific ultraviolet absorption of 1.37 (1 mg/ml) at 280 nanometer and pH = 7.0 (Carl Zeiss spectrophotometer). Soya bean trypsin inhibitor was obtained from Wortington (lot no. 56B449), Sigma (lot No. 85C8057) and the British Drug Houses (lot No. 2985980). The purity was checked by amino acid analysis (Durrum) and continuous ultraviolet spectrum (230–350 nm range) and compared to literature data [22]. Inhibitor protein content was estimated, assuming specific optical density of 1.0 for a 1 mg/ml solution at 280 nm. Either the Wortington preparation (0.6 mg inhibitor/mg product) or the Sigma vial (0.72 mg inhibitor/mg product) was used. ε-amino-*n*-caproic acid (lot No. 124C0056), para-amino-benzamidine (lot No. 15C0015) and imidazole (lot No. 53C5500) were supplied by the Sigma Corporation.

U shaped wells containing agglutination plates were obtained from Linbro Chemicals, New Haven (Code IS-MCR-96).

Enzymes and buffers

Trypsinolysis was performed in (0.01 M Kaliumphosphate–0.15 M NaCl; pH = 7.3) (PBS), using Sigma trypsin (lot no. F 8253) with 11,400 B.A.E.E. units/mg. α-Chymotrypsin treatment was done in PBS using a Wortington vial (lot no. 540404) with 50.1 units/mg. Proteolytic activity of pronase (Calbiochem- lot no. 10118-units non-specified) was tested in (0.1 M Borate–0.05 M HCl–2 mM $CaCl_2$; pH = 7.5) buffer. Pepsinolysis was done in (1 mM glycin-0.15 M NaCl; pH = 3.2) buffer (Sigma, lot no. P 7012–4100 units/mg).

Urokinase (Warner–Lambert Research Institute, Wa-13,497-1, lot No. 1) was obtained as a lyophilized material containing 300,000 CTA units, kept frozen and diluted in phosphate buffered saline just before use. Assuming that 200,000 CTA units correspond to 1.5 mg active protein [23], we expressed the enzyme concentration in mg/ml quantities.

Concanavalin A agglutination assay

In Linbro plates, 0.05 ml PBS was pipetted and twofold consecutive dilutions were made by adding 0.05 ml from an original 1 mg/ml Con A solution in PBS buffer. After an incubation period of 30 min, 0.05 ml of the target cell suspension was added at a concentration of 10^8 erythrocytes/ml and agglutinations were scored after 1 hr at 37°C. Every experiment was performed in triplicate.

Experimental animals and tumors

Mice and tumors. For all studies, two strains of mice and three tumors were used. We chose to work with lymphoid L1210 leukemia because it offers an experimental model that is well documented in literature and allows activity prediction against leukemia [24]. We also worked with the lymphocytic P388 leukemia and the well known Ehrlich ascites carcinoma.

For each experiment, 10–20 mice were used. Mean survival time ± the standard deviation (S.D.) of the treated mice and the control mice, as well as the dosage and timing of each experiment are specified in the tables.

Survivors on day 90 after tumor graft were considered as long-term survivors, killed and were not used in the final evaluation of the mean survival time. A 25% increase in mean survival time of treated mice over controls was considered significant and supported by a single tailed Fisher *t*-test.

CDF_1 mice. Inbred (BALB/c × DBA/2) F_1 animals were supplied by Charles River Breeding Laboratories. Those mice were stored at least for 3 weeks in an isolation room before we started experimenting. Female CDF_1 mice (BALB/c female × DBA/2) F_1 animals weighing 19–23 g were used. L1210 lymphoid leukemia and P388 lymphocytic leukemia were originally obtained from Mr. I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.).

Those tumors are maintained in DBA/2 mice by weekly intraperitoneal (i.p.) inoculation.

L1210 or P388 ascites were aspirated from DBA/2 mice and a suspension was made in Hank's balanced salt solution; 10^4 cells in 0.1 ml solution were injected intraperitoneally to obtain the ascitic form of leukemia. Animals were randomized into test groups and control groups.

BALB/c mice. An hyperdiploid line of the Ehrlich ascites tumor was originally obtained from Dr. Maisin. This tumor has been transplanted for years by Mr. A. Cordier in inbred BALB/c mice by weekly passage of 10^7 tumor cells to intact mice. Either male mice weighing minimum 19 g or female mice weighing at least 18 g were used.

Ehrlich ascites tumor cells were aspirated from BALB/c mice and diluted in phosphate buffered saline, 0.1 ml of the suspension was injected i.p. to obtain the ascitic form of the carcinoma. Animals were randomized into test groups and control groups.

RESULTS

1. *Effect of proteolysis on the agglutinability of rabbit erythrocytes by Concanavalin A*

Rabbits were obtained from a local breeder and blood from the ear vein was collected and pooled vol/vol in heparin containing Alsever solution. After repeated washings and centrifugations at 3015 × ***g*** for 20 min, at 4°C, the erythrocyte pellet was resuspended in the appropriate buffer ten times the original blood volume. Under frequent shaking, the cell suspensions were incubated at 37°C and an aliquot of the enzyme stock solution was added to a final 0.1 % enzyme concentration. At 10, 20, 40, 60, 90 and 120 min of incubation, samples were removed from the reaction mixture, centrifuged and resuspended in PBS buffer.

In Linbro plates, cells (10^8 erythrocytes/ml) were assayed for agglutination by 1 ml/ml Con A solution.

In Table 1, mean values of the Con A agglutination titer are expressed in $\log_2$ units as a function of the incubation period with the different enzyme solutions. We observed that, for each enzyme tested, long incubation periods (60–120 min) yield comparable agglutination patterns, increased over untreated controls. Agglutinability of cells exposed to trypsin was comparable to chymotrypsin treated erythrocytes. Incubation with pepsin was less effective and prolonged incubation with pronase resulted in lysis of many cells after 90 min.

2. *Effect of trypsin or urokinase on the agglutinability of mouse erythrocytes by Con A*

BALB/c mice were bled in the retro-orbital plexus and blood was collected in heparin containing Alsever solution..

After repeated washings and centrifugations at 297 × ***g*** for 10 min, at 4°C, the erythrocyte pellet was resuspended at 2.10^9 cells/ml in PBS buffer. One twentieth of a millilitre of the suspension was incubated for 30–60 min with 0.1 ml of trypsin (final concentration: 0, 0.02, 0.2, 2.0 mg/ml) or urokinase concentrations (final concentration: 0, 0.02, 0.2 mg/ml) to which

Table 1. *Effect of protease on the agglutinability of rabbit red blood cells by Concanavalin A*

	$\log_2$ agglutination titer as a function of the incubation time (min)					
Enzyme	0	20	40	60	90	120
Trypsin	9.5	14	15	15.5	16	15.5
Pepsin	9.5	9.5	9.5	11.5	11.5	12
α-Chymotrypsin	9.5	9.5	12.5	16.5	16	15.5
Pronase	9.5	16.5	15.5	13.5	8.5	NT
No. (blank)	9.5	NT	NT	NT	NT	9.5

Rabbit erythrocytes were treated by 0.1 % enzyme solutions and samples were removed at different incubation times.

Agglutinability by 1 mg/ml Con A were scored after 1 hr at 37°C.

NT = not tested.

Table 2. Effect of incubation or effect of a single administration of SBTI on Ehrlich ascites tumor growth

Schedule number	Trypsin inhibitor concentration	Treatment (day)	MST ± S.D. (days)
A1	0.2 mg/ml	0	21.0 ± 1.94
A2	0.050 mg/ml	0	25.0 ± 1.99
A3	0.010 mg/ml	0	23.5 ± 0.6
A4	0	–	21.6 ± 0.4
B1	50 mg/kg	1 hr before transplant	22.33 ± 1.29
B2	50 mg/kg	24 hr after transplant	22.0 ± 1.32
B3	0		22.0 ± 0.70

In schedules A1–A4, 4.10^6 Ehrlich cells were incubated vol/vol (30 min—0°C) with different solutions of SBTI and 2.10^5 cells were grafted to BALB/c mice intraperitoneally on day 0.

In schedules B1–B3, BALB/c mice were inoculated intraperitoneally with 2.10^5 Ehrlich cells on day 0 and treated as indicated.

MST ± S.D. = Mean survival time ± standard deviation.

0.2 ml of pooled BALB/c plasma was added. After the incubation, the cells were washed, centrifuged at 297 × *g* for 10 min at 4°C, adjusted to 10^8 cells/ml and tested for Con A agglutinability. As seen in Table 2, agglutinability was shown to be dependent on the enzyme concentration. Very likely, enzyme activity at low doses could be inhibited by earlier described serum protease inhibitors, such as the α_1 antitrypsin protein [25].

3. *Agglutinability of blood taken from Ehrlich ascites tumor bearing mice*

Eleven intact male BALB/c mice were inoculated intraperitoneally with 2.10^5 Ehrlich ascites cells on day 0.

On days 0, 5, 9, 15 and 18, blood from the retro-orbital plexus was collected and stored at 4°C in heparin containing Alsever solution and 0.25 ml blood was added to an aliquot of 0.15 ml heparin containing Alsever solution. After extensive washings and centrifugations at 297 × *g* for 10 min, 4°C, erythrocytes were adjusted to 2.5 × 10^8 cells/ml in PBS buffer.

In Linbro plates, target erythrocytes were tested for agglutinability by 2 mg/ml Con A solution. Mean values and the standard deviation were recorded as indicated in Fig. 1. Erythrocytes of intact BALB/c mice (2.5 × 10^8/ml) were treated with 0.1% trypsin in PBS for 1 hr, washed twice and centrifuged at 297 × *g* for 10 min at 4°C. They were assayed in parallel with untreated erythrocytes for Con A agglutinability. As seen in Fig. 1, we observed that agglutinability increases with tumor age and reaches agglutination titers comparable to those of *in vitro* enzyme treated cells, suggesting a similar *in vivo* proteolytic activity in Ehrlich ascites tumor bearing mice. We also found that after grafting intraperitoneally 10^4 L1210 or P388 tumor cells in CDF_1 mice on day 0, $\log_2$ agglutination titers increased from 8.63 ± 0.61 (day 0) to 9.19 ± 0.44 on day 10. We assume that the tumor grew too quickly and did not persist long enough to show good detectable effects.

4. *Influence of incubation or administration of SBTI on Ehrlich ascites tumor growth*

First, we looked for a direct interaction of SBTI with non-malignant cells. We observed that high dose of trypsin inhibitor (10 mg/ml) could agglutinate mouse splenocytes (Sigma vial: $\log_2 = 2.0$; BDH vial: $\log_2 = 9.0$) or mouse erythrocytes (Sigma vial: $\log_2 = 0.0$; BDH vial: $\log_2 = 7.5$) irrespective of the incubation temperature (4°C and 37°C). Since we found that

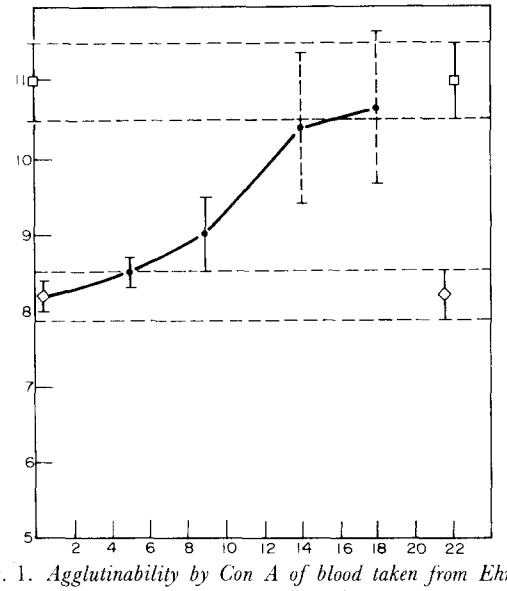

Fig. 1. *Agglutinability by Con A of blood taken from Ehrlich ascites tumor bearing mice. Intact BALB/c mice were inoculated intraperitoneally with* 2.10^5 *Ehrlich cells on day* 0. *On days* 0, 5, 9, 15 *and* 18, *blood from the retro-orbital sinus was collected and agglutinability of erythrocytes by Concanavalin A was scored* (○) *and compared to trypsin treated* (□) *or untreated erythrocytes of intact mice* (◇). (*Agglutination titer* ± *the standard deviation.*) *Abscis* = $\log_2$ *haemagglutination titer Ordinate = tumor age* (*days*).

those commercial trypsin inhibitors are pure at 60–72%, it seems logic to ascribe this property at least to a contaminating agent in the preparation. This has led us to choose the Sigma product for further experiments.

Then, we investigated if there was a direct (passive) cytotoxic effect of SBTI on 4.10^6 Ehrlich cells in PBS buffer in a vol/vol ratio at low temperature (0°C) with SBTI preparations of 0.4 mg/ml, 0.1 mg/ml or 0.02 mg/ml and a blank (control).

We assumed that a 30-min incubation period is sufficiently long to allow interaction of inhibitor with cell membrane proteins even in the presence of reduced metabolic activity (0°C). Grafting those incubated cells to intact mice did not extend mean survival time of those mice as indicated in Table 3. We also observed that a 50 mg/kg-single administration dosage of SBTI was ineffective against 2.10^5 implanted Ehrlich ascites cells. Then we applied a chemotherapeutic trial after an initial tumor graft on day 0. For 2.10^4 grafted cells, we found that a 20 mg/kg inhibitor dose resulted in 70% of long-term survivors, when given on days 1–5 the mean survival time of treated, but tumor bearing mice, was significantly prolonged, as recorded in Table 4. For 2.10^5 grafted cells, the percentage of long-term survivors varied from 40–0% of the mice and depended on the treatment schedule when 50 mg/kg were administered repeatedly as indicated in Table 4. Other treatment schedules were also therapeutically effective but a treatment started on day 7 after tumor grafting was ineffective.

Finally, we tried to improve chemotherapeutic effects by a combination with immunotherapy. Assuming an interaction of the inhibitor with a membrane protease, we hoped to kill SBTI coated tumor cells immunologically. Antibodies to trypsin inhibitor were elicited in rabbits by subcutaneous injection of 0.5 mg of protein in PBS buffer emulsified in complete Freund adjuvans. Animals were boosted after one month with three injections of 0.5 mg within two days interval and bled 14 days later.

Table 3. Con A agglutinability of mouse erythrocytes incubated with urokinase or trypsin

Enzyme + concentration		Log_2 haemaggl. at 30 min	Log_2 haemaggl. at 60 min
Trypsin	0.002 mg/ml	9.0	9.0
	0.02 mg/ml	9.0	9.0
	0.2 mg/ml	9.0	11.0
	2.0 mg/ml	10.5	14.0
	0 mg/ml	8.5	9.0
Urokinase	0.002 mg/ml	8.5	8.5
	0.02 mg/ml	8.5	8.5
	0.2 mg/ml	9.5	10.5
	0 mg/ml	8.5	8.5

Mouse erythrocytes suspended in mouse plasma were tested for agglutinability by Con A after incubation at 37°C in presence of the enzymes urokinase or trypsin.

Table 4. Effect of administration of SBTI on Ehrlich ascites tumor growth

Schedule of treatment (injection days)	Number of grafted tumour cells	Treatment dosage (mg/kg)	Mean survival time ± standard deviation (days)	% Long term survivors (day 90)
C1 1, 2, 3, 4, 5	2.10^4	20	59.3 ±6.76+	70
C2 1, 3, 5, 7, 9	2.10^4	idem	60.7 ±7.50+	62.5
C3 7, 8, 9, 10, 11	2.10^4	idem	45.00±8.06+	30
C4 1, 4, 7, 10, 13	2.10^4	idem	48.4 ±8.10+	40
C5 no (control mice)	2.10^4	idem	25.9 ±0.90	0
D1 1, 2, 3, 4, 5	2.10^5	50	37.29±8.46+	20
D2 1, 3, 5, 7, 9	2.10^5	idem	21. ±0.96	10
D3 7, 8, 9, 10, 11	2.10^5	idem	19.33±0.75	0
D4 1, 4, 7, 10, 13	2.10^5	idem	23. ±1.73	40
D5 no (control mice)	2.10^5	idem	22.5 ±1.05	0

All BALB/c mice received a transplant of Ehrlich ascites cells intraperitoneally on day 0.
Significant (*t*-test) at $P<0.01$ *level* (+).

Analysis of the rabbit sera revealed the presence of antibodies precipitating with SBTI in 1% agar supplemented with 4% polyethyleneglycol. Those heterologous sera were repeatedly absorbed with pooled mouse blood cells to remove cytotoxic and agglutinating antibodies, following the technique of Boyse *et al.* [26]. Intact randomized BALB/c mice received a tumor graft of 2.10^5 cells on day 0 and they were treated on days 1, 4, 7, 11 and 16 with low doses of inhibitor (5 mg/kg).

Untreated control mice died with a mean survival time of 21.5 ± 1.93 days. Mice treated intraperitoneally died after 19.37 ± 1.92 days (11% of long-term survivors), whereas all mice treated intravenously died after 21.7 ± 1.37 days. Mice which received i.p. treatment followed by intraperitoneal inoculation of 0.2 ml antiserum lived $26.9 + 4.57$ days (11% long-term survivors), whereas all mice treated i.v. and inoculated intraperitoneally with 0.2 ml of antiserum died after 18.75 ± 4.59 days. In a subsequent approach, mice were directly vaccinated with SBTI in complete Freund adjuvans, but this immunochemotherapeutic experimentation did not yield any improved outcome.

5. *Comparison between antitumor activity of trypsin inhibitor and urokinase inhibitors on murine ascites tumor growth*

In a last trial, we tested, with equal pharmacological dosages, different inhibitors of urokinase as well as SBTI for activity against L1210 and P388 leukemia and Ehrlich carcinoma.

All tumor grafted mice received intraperitoneal treatment on days 1–5 with 20 mg/kg doses. Under the present conditions, it was observed that neither inhibitor affected L1210 or P388 growth, whereas Ehrlich ascites carcinoma was inhibited by ε-amino-*n*-caproic acid (70% of long-term survivors), para-aminobenzamidine (30% of long-term survivors) and by SBTI (10% of long-term survivors), as seen in Table 5.

DISCUSSION

The conceptual ideas dealing with the correlation of tumor growth, metastasis and proteolytic activity are complex. It is established now that tumor cells, unlike their parent normal cells, lyse clotted plasma substrates [5]. This fibrinolytic activity is generated by the interaction of serum plasminogen (PA) [7] found in the supernatants of tumor cell cultures [8]. We hypothesize that PA may play a crucial role in monitoring invasiveness in other tissues and organs, a mechanism in common with PA of some non-malignant cell lines, such as macrophages. Therefore, we tried to determine the biological effects of inhibiting PA activity in tumor bearing mice. Very little is known about PA activity of P388 or L1210 cells but Funahara *et al.* found that PA could be extracted from Ehrlich cells [17]. Leblanc and Bach were

Table 5. *Effect of SBTI and urokinase inhibitors on murine ascites tumor growth*

Agents	Tumor and number of injected cells		
	Ehrlich carcinoma (2.10^5)	P 388 (10^4)	L1210 (10^4)
Para-amino-benzamidine			
MST ± S.D.	27.63 ± 7.73	12.3 ± 0.5	11.62 ± 0.52
%C	30.	0.	0.
ε-amino caproic acid			
MST ± S.D.	32.0 ± 12.63	12.0 ± 0.0	11.0 ± 0.0
%C	70.	0.	0.
SBTI			
MST ± S.D.	23.75 ± 3.28	12.0 ± 0.0	11.25 ± 0.46
%C	10.	0.	0.
Imidazole			
MST ± S.D.	22.5 ± 3.06	12.37 ± 0.74	11.75 ± 0.46
%C	0.	0.	0.
Control mice			
MST ± S.D.	22.89 ± 3.86	12.36 ± 0.93	11.82 ± 0.6
%C	0.	0.	0.

All mice received a tumour transplant on day 0, intraperitoneally.
On days 1, 2, 3, 4, 5 mice were treated intraperitoneally with a 20 mg/kg dosage.
Mean survival time ± the standard deviation (MST ± S.D.) and the percentage (%C) of long-term survivors (day 90) are indicated.

unable to detect any PA activity in Ehrlich ascites fluid but observed that Ehrlich ascites tumor growth *in vivo* is accompanied by a kinin forming protease system that closely interrelates with both the fibrinolysin and blood coagulation systems [27]. Working with a standardized agglutination technique, we found that agglutinability of erythrocytes by Con A was increased after exposure to protease but with quantitative differences among the enzymes studied. Similarly, agglutinability of mouse erythrocytes was increased when incubated with trypsin or urokinase in the presence of mouse plasma. Taking advantage of this test, we observed that erythrocytes taken from Ehrlich tumor bearing mice display higher agglutinability by Con A, increasing with tumor age and suggesting the presence of a similar proteolytic activity *in vivo*. Agglutination patterns of P388 or L1210 tumor bearing animals were not considerably elevated.

Search for a direct cytotoxic effect of SBTI (passive absorption) by a short time incubation with Ehrlich cells and grafting those incubated cells to intact mice, revealed that mean survival time of treated mice was not prolonged over control mice.

However, those *in vivo* results require to be extended by *in vitro* SBTI coculture studies. Applying chemotherapy to tumor bearing mice clearly shows that for 20,000 initially grafted tumor cells, inoculation of 20 mg/kg SBTI exerts a chemotherapeutic effect, up to 70%, for different treatment schedules and significantly increases mean survival time over control mice. For 200,000 tumor grafted cells, treatment by a 50 mg/kg dose still resulted in 40% of long-term survivors. Time spaced schedules proved to be most efficient. In a comparative study, we tried to inhibit growth of different murine ascites tumors by SBTI and inhibitors of urokinase. On an equally pharmacological basis, we found that treatment of Ehrlich ascites carcinoma by ε-amino-*n*-caproic acid, para-aminobenzamidine and SBTI resulted respectively in 70, 30 and 10% of long-term survivors, whereas neither product affected L1210 or P388 leukemia growth. The fact that SBTI did not inhibit murine leukemia growth does not exclude prognosis for human leukemia. It is known that human lymphoblastic leukemic cells are often coated by tumor specific IgG fragments, which suggests that protease activity operates *in vivo* [28].

We believe that we can attribute our results to a direct inhibition of PA activity *in vivo*. However, we do not exclude the possibility that inhibition of degradation of fibrinogen by plasmin may favor specific anti-tumor responses in the weakly immunogenic Ehrlich tumor bearing mice (inhibition of formation of immunosuppressive fibrin microparticulates) and not in the poorly immunogenic L1210 or P388 tumor bearing mice.

Clinical as well as laboratory studies show that leukemic cells and solid tumor cells tend to form clumps of two or more cells formed on fibrin substratum, which are important in metastasis [29]. A solid tumor needs a network of blood capillaries to grow beyond a certain size [30]. Some reports indicate that a fibrin substrate is essential for capillaries to grow in the tumor tissue, whereas others indicate that fibrin is found at early metastatic sites [31–33]. Further studies can determine the effects of administration of those trypsin and urokinase inhibitors on metastatic leukemia and solid tumor growth. We hypothesize that PA may play a crucial role in monitoring invasiveness in other tissues and organs. We also hypothesize that the administration of SBTI may favor specific immunotherapeutic trials, when given intratumorily.

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Studies on the Selective Toxicity of Guanazole in Mice*

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Abstract—*Guanazole (3,5-diamino-1,2,4-triazole (GZ), an antileukemic agent effective against acute myelocytic leukemia, seems to exert its action through inhibition of ribonucleoside diphosphate reductase. Using recently developed technique for drug infusion in unrestricted mice, 5–30 mg/ml guanazole was infused at a rate of 0.2–0.3 ml/hr in normal mice and mice bearing leukemia L1210 or a subline resistant to guanazole (L1210/GZ). Incorporation of ^{14}C-uridine (UR) into DNA was considerably reduced in spleen, thymus and bone marrow; incorporation of ^{3}H-thymidine was reduced to a much greater extent in these tissues. The inhibition of precursor incorporation was dependent on the concentration of guanazole used and was somewhat correlated to the intracellular drug concentration in different tissues. This inhibition was in the following order: L1210 spleen > normal spleen > L1210/GZ spleen. Analysis of free intracellular ribonucleotide pools indicated the presence of a high IMP and a low ATP pool in normal spleen, and a moderate IMP and a high ATP pool in spleen infiltrated by cells from either leukemia; this profile resembled that observed in intraperitoneally growing L1210 cells. Guanazole infusion resulted in the reversion of pools profile from that characteristic of L1210 spleen to that of normal spleens. The morphological form and microscopic characteristics of L1210 spleen from mice treated with GZ also resemble those of spleen from non-leukemic mice.*

Study of in vitro uptake of guanazole in leukemia L1210, indicated that the transport occurred by a passive diffusion process. In the subline, L1210/GZ, the initial rate of uptake was 1/3rd to 1/4th of that in the parent line. This suggested that a generalized change in membrane architecture is likely to have accurred in L1210/GZ.

The results of this study are consistent with the idea that in different tissues in mice, the mechanism of action of guanazole is dependent upon inhibition of ribonucleotide reductase, whereas its selectivity of action is dependent upon differences in uptake.

INTRODUCTION

GUANAZOLE (3,5-diamino-1,2,4-triazole, NSC 1895) (GZ) is an agent having activity against mouse leukemia L1210 [1]. It has also been found to be effective against acute myelocytic leukemia [2, 3]. The complete remission was obtained with the drug in phase I and II clinical trials in between 10 and 20% of the cases.

The major limitation in therapy with GZ[2] is its hematologic toxicity [2, 3]. However, in a limited number of cases, the response to GZ was remarkably selective [2]. Since the drug is not metabolized by mouse [4], rat [5], or human [6] and since the differences in response between target host and tumor cells were quantitative, the selectivity of action may reside in a differential drug distribution between host and tumor cells or in differential sensitivities of cellular target sites. The only known biochemical mechanism of action of GZ is the inhibition of ribonucleoside diphosphate reductase [7]. The accumulation of cells in the G_1/S boundary of the cell cycle observed after *in vitro* treatment with GZ [8] is consistent with the above-mentioned mechanism.

After methodology of continuous i.v. infusion of drugs into unrestrained mice was developed in this laboratory [9], it became feasible to study the basis for the selective toxicity of GZ *in vivo* under treatment conditions similar to those employed for treatment of patients [3]. Correlations between drug concentration, ribonucleotides pool sizes and incorporation of nucleic

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acid precursor into RNA and DNA in different mouse tissues were drawn based on the results of this investigation.

MATERIAL AND METHODS

Animals and tumor in vivo

DBA/2J female mice weighing between 18–20 g were obtained from Jackson Laboratories, Bar Harbor, Maine. Since the last 10 yr, leukemia L1210 has been maintained in these mice in this laboratory by weekly i.p. transplantation (1×10^6 cells/mouse).

During the course of these studies, a subline of leukemia L1210 resistant to GZ (L1210/GZ) was developed over a period of 6 months by treating tumor-bearing mice from day 1 through day 5 with gradually increasing doses of GZ (200–1000 mg/kg, i.p.) and utilizing the surviving ascites tumor cells for subsequent transplantation. Treatment of mice bearing the L1210/GZ with GZ did not result in an increase in survival time greater than 5% of the controls.

For studies involving continuous infusions of GZ into leukemic mice, 10^6 L1210 cells were inoculated i.v., rather than i.p. and at specified times thereafter, the drug was infused continuously as described below.

In vitro *drug uptake*

Leukemia L1210 or L1210/GZ cells were obtained from the peritoneal cavity of DBA/2J mice inoculated i.p. with 2×10^6 cells/mouse, 4 days prior to cell collection. Harvested cells were washed with RPMI 1640 medium to remove the contaminating ascites fluid and red cells. The cells (1×10^7/ml, 5 ml/tube), were then suspended in RPMI medium containing 10% dialyzed fetal calf serum and incubated at 37°C with different concentration of ^{14}C-GZ for varying time. Total uptake by the cells in wet pellet was determined as the amount of radioactivity in the wet pellet minus that in fluid present in the extracellular space. The corrections for the extracellular space volume and radioactivity were determined by standard ^{3}H-inulin methods. The details of the procedures used have been described elsewhere in detail [10].

Intravenous infusion procedure

Mice were temporarily restrained and the tail vein was cannulated with saline-filled polyethylene tubing (Intramedic PE No. 10, Clay Adams Co.) through a cut made with a surgical suture needle (Ethicon, Type B No. 682). Proper positioning of the cannula was routinely verified by the backflow of blood. The other end of the tube was connected to a Luer Lock syringe by means of a No. 27 gauge hypodermic needle. The cannula was protected by a 75×12 mm polystyrene tube and the mouse was released in the cage with the infusion assembly connected to a Harvard infusion pump [9]. In this way, it was possible to infuse drug for several days without visible trauma to the animals. Groups of mice were infused with saline or GZ solution for varying periods of time at a flow rate of 0.3 ml/hr.

Incorporation of radiolabeled nucleosides

At the termination of infusion, ^{14}C-uridine of a specific activity of 2.5 μCi/0.4 μmole/0.2 ml and ^{3}H-thymidine of a specific activity of 25 μCi/0.1 μmole/0.2 ml were immediately injected through the polyethylene cannula and the mice were sacrificed 15 min thereafter. In some experiments, ^{3}H-uridine (UR) and ^{14}C-thymidine (TdR) were used without any change in the quantities injected. Tissues were rapidly removed and frozen in liquid nitrogen. Tissue homogenates were then prepared in cold 6% perchloric acid (PCA) by 10 strokes with a Ten-Broeck homogenizer. Bone marrow cells were recovered from both femurs by rinsing the marrow cavities with 1 ml saline per femur. These cells were also treated with 6% PCA.

The homogenates were centrifuged at 1000 ***g*** for 10 min at 4°C. The PCA-soluble supernatant fraction containing nucleosides and nucleotides, as well as GZ, was assayed for total radioactivity and for GZ.

To remove residual soluble matter, the PCA pellet containing nucleic acids and other macromolecules was washed 3 times with 5 ml of cold 6% PCA. DNA and RNA were separated by the method of Schneider [11]. This procedure involved alkaline hydrolysis of RNA with 1 ml of 1N NaOH at 37°C for 1 hr followed by neutralization and extraction of RNA nucleotides with cold PCA. DNA nucleotides were extracted from the residue by treatment with PCA at 70°C for 15 min. Aliquots of RNA and DNA were counted for radioactivity. RNA content was determined by the orcinol reaction [11] and DNA by a modification of Burton's method [12] using 0.6 mM paraldehyde instead of acetaldehyde [9].

Guanazole was determined colorimetrically by a procedure involving diazotization and coupling with diphenylamine [13].

Determination of ribonucleotide pool sizes

Purine and pyrimidine ribonucleotides in spleen and in L1210 cells obtained from i.p. ascites tumor were separated by diethylamino-

ethyl (DEAE)-cellulose column chromatography and quantitated as described previously [14]. Identification of components was based on their elution position on the calibrated column, and by absorbance ratio at 280/254 nm at pH 4.4 and 12. Spleens were removed under minimum anesthesia and placed into liquid nitrogen. A high speed of tissue removal was essential to minimize the breakdown of nucleoside triphosphates [15, 16]. Labeled ATP added to tissues as a marker prior to extraction with PCA was recovered quantitatively indicating that no breakdown of triphosphates occurred during tissue processing.

Measurement of radioactivity

Radioactivity was determined in a Packard liquid scintillation counter by counting up to 1 ml aliquot of the aqueous samples in 1 ml of a scintillation counting fluid containing 0.6% w/v PPO, 0.06% dimethyl POPOP and 15% Beckman Biosolv 3 in toluene. The counts were corrected for quenching using the external standard ratio method [17].

RESULTS

Differential effect of GZ on the incorporation of nucleic acid precursors in different tissues in mice

The effects of GZ on the incorporation of TdR and UR into nucleic acids of mouse tissues are summarized in Table 1. Following 18 hr of continuous infusion, the drug produced greater inhibition of ^{14}C-UR incorporation into DNA than into RNA. The maximum inhibition occurred in spleen, thymus and bone marrow cells. No inhibition occurred in kidney and liver tissues. In the liver, a stimulation of incorporation into nucleic acids was apparent. Furthermore, GZ was more inhibitory to the incorporation of ^{3}H-TdR into DNA than that of UR into the DNA of lymphoid and hemopoietic tissues. In other tissues either no effect or a stimulation of incorporation was observed. In the experiments involving both UR and TdR, no noticeable differences were observed between total acid-soluble pools of corresponding tissues from control and GZ-treated mice. This suggested that GZ has no effect on the transport of nucleotides *per se*.

The GZ effect on thymidine incorporation could not be reversed when an equimolar mixture of the other three unlabeled deoxyribonucleosides was injected together with thymidine.

The inhibition of UR and TdR incorporations into DNA of lymphoid and hemopoietic tissues appeared to be somewhat related to the intracellular GZ concentrations (Table 1). Consistent results were obtained when 5 and 30 ml/mg GZ solutions were infused for 1 hr at which time, steady-state level of GZ was presumably reached in plasma [9]. Thus incorporation of uridine into DNA of spleen and thymus decreased respectively from about 25 to 12 and 43 to 22% of the control on increasing GZ concentration from 5 to 30 mg/ml. Under the same conditions, incorporation of uridine into

Table 1. *Distribution of GZ in non-leukemic DBA/2J mice following* 18 hr *i.v. infusion and effects on the incorporation of uridine and thymidine into nucleic acids of various tissues*

Tissue	Guanazole μg/g tissue	Uridine incorporated into DNA (% of the control)	Uridine incorporated into RNA (% of the control)	Thymidine incorporated into DNA (% of the control)
Spleen*	65.3 ± 7.6	21.6 ± 2.6	49.2 ± 7.4	2.1 ± 0.1
Thymus*	63.8 ± 7.9	40.4 ± 4.5	84.1 ± 4.1	11.3 ± 0.7
Bone Marrow*	—	51.6 ± 12.2	87.5 ± 3.3	6.2 ± 0.4
Heart†	31.4 ± 6.4	82.0 ± 4.9	99.1 ± 6.4	191.1 ± 11.5
Brain	22.7 ± 2.7	85.1 ± 10.2	110.6 ± 9.5	187.3 ± 19.5
Kidney†	45.1 ± 10.2	105.2 ± 7.9	100.3 ± 7.1	101.0 ± 7.6
Liver†	34.8 ± 4.2	130.5 ± 21.3	121.3 ± 8.3	132.6 ± 8.9

Guanazole (5 mg/ml) was infused i.v. for 18 hr at the rate of 0.3 ml/hr. Control animals received infusion of normal saline. Specific incorporation of ^{14}C-uridine into DNA and RNA was determined and expressed as % of control values. Incorporation ^{3}H-thymidine into DNA was also expressed as % of control. The average values for incorporation of uridine into DNA (dpm/mg DNA) of spleen, thymus, bone marrow, heart, brain, kidney and liver from saline-treated mice were: 28,654; 1,475; 37,142; 6,931; 1,218; 6,325 and 13,230, respectively and those into RNA (dpm/mg RNA) were: 12,291; 9,311; 4.574; 23,149; 8,939; 12,245 and 13,191, respectively.

*†Each value is an average based upon either (*) 6 control and 6 drug-treated animals or (†) 4 control and 4-drug-treated animals ± standard deviation.

RNA did not change in the case of thymus and decreased from 52 to 41% of control in the case of spleen. With this treatment, intracellular concentration of GZ increased from 60 ± 10 to 331 ± 20 mg/g wet weight of the spleen respectively (2 mice/group).

Drug uptake in L1210 and L1210/GZ in vitro

Since the data obtained with GZ infusion (Table 1) suggested that the tissue-selective effects of GZ is related to differential GZ uptake,

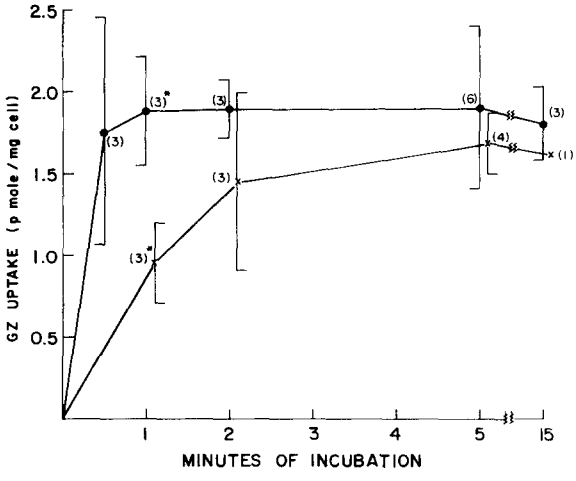

Fig. 1. In vitro *uptake of* ^{14}*C-GZ at varying periods of time. L1210 (●) or L1210/GZ (×) cells obtained from the i.p. cavity of DBA/2J female mice on the* 4th *day after transplantation of* 2 × 10^6 *cells, were incubated with* ^{14}*C-GZ* (4.0 μM) *at* 37°C *as described in the text. Incubation with GZ was carried out for varying periods of time up to* 15 min *and the drug uptake per mg net cell weight was determined.*

Each point is an average of the number of experiments indicated in parenthesis; vertical bars indicate standard deviation. All the uptake values for the L1210/GZ cells, have been shifted to the right by 0.1 min *in order to accommodate the vertical bars. *Values significantly different at* $P = 0.002$ *using Student's t-test.*

it was of interest to compare the GZ uptake in L1210/GZ cells. The course of uptake of GZ in both cell lines is shown in Fig. 1. The initial rate of GZ uptake was very rapid in both cell lines although in L1210 cells, it was about 2–3 times greater than in L1210/GZ cells. Differences between the two lines in the total amount of GZ taken up decreased considerably within 5 min and the uptake seemed to remain constant thereafter up to 15 min. At that time point, the intracellular concentration in L1210 cells was about 75% of that in the extracellular water, assuming that all the GZ was distributed in intracellular water [about 60% net weight of the cells [6]. In the case of L1210 cells, the initial rate of uptake determined at 1 min after drug addition did not show any saturation over a 250-fold range of extracellular drug concentration (Table 2). It may be mentioned that throughout

Table 2. Effect of drug concentration on the in vitro *uptake of* ^{14}*C-GZ by mouse leukemia L-1210*

GZ concentration (μM)	Uptake (pmole/mg cell/min)	I/E ratio
4.0	1.9 ± 0.5 (6)	0.79
14.0	7.5 ± 0.1 (2)	0.92
100.0	59.6 ± 7.1 (2)	0.99
1000.0	514.5 ± 62.7 (2)	0.86

DBA/2J female mice were inoculated i.p. with 2 × 10^6 L-1210 per mouse on day 0. On day 4, the mice were sacrificed and the leukemic cells were harvested, washed and incubated at 37°C with varying concentrations of ^{14}C-GZ as described in Materials and Methods, for 1 min. The uptake was determined as described and I/E was calculated assuming 60% of cell volume as total intracellular water and the specific gravity of the cells as 1.0.

Each value is an average of the number of observations ± S.D. or 0.5 range in the case of two observations. Number of observations are shown in parentheses.

the time-course of this study, the cell viability as determined by trypan blue exclusion remained 98–100% for both the cell lines.

Effect of GZ in mice bearing leukemia L1210

A comparison of the effect of GZ on the survival of DBA/2J-female mice given L1210 and L1210/GZ leukemia i.p., is shown in Table 3. The growth of L1210/GZ appeared to be somewhat slower than that of L1210 as inferred from the increased survival of the corresponding untreated mice. This is consistent with the marked increase in immunogenicity of this tumor [18]. Drug treatment did not have any significant effect on the survival of mice bearing the resistant leukemia.

When DBA/2J female mice were injected i.v. with 10^6 L1210 cells, their average survival was 4–5 days. On autopsy, the spleen of these mice was found to be enlarged and increased in weight from 60–70 mg to 250–300 mg, and leukemia cell colonies could be seen. No ascites fluid

Table 3. Effect of guanazole treatment on survival of leukemia-bearing mice

Dose of GZ (mkg × 5)	Average survival (days) ± S.D. L-1210	L-1210/GZ
Control	8.6 ± 0.4	9.2 ± 0.5
450	11.0 ± 1.2	9.6 ± 0.5

DBA/2J female mice were inoculated i.p. with L-1210 or L-1210/GZ cells (1 × 10^6/mouse).

Groups of 10 mice were either treated with saline (control) or GZ once daily for 5 days from day 1 onwards and their survival was observed.

Table 4. Effects of guanazole infusion for 1 hr *on incorporation of* ^{14}C*-thymidine and* ^{3}H*-uridine into spleen DNA of leukemic and non-leukemic mice*

Tumor in spleen	Infused guanazole (mg/ml)	Guanazole (μg GZ/μg DNA)	TdR incorporation into DNA (% of the control)	UR incorporation into DNA (% of the control)
None	5	27± 3	44.0± 3.5	78.1±1.9
	30	85± 6	10.1± 3.2	35.3±2.0
L1210	5	36± 6	19.1± 1.0	75.8±9.7
	30	103± 7	7.2± 4.1	31.9±6.1
L1210/GZ	5	11± 3	79.6± 9.8	88.8±8.4
	30	41±15	66.3±10.2	72.9±1.5

GZ (5 or 30 mg/ml) was infused for 1 hr at rate of 0.2 ml/hr (control mice were infused with saline). A mixture (0.2 ml) containing 2.08 μCi/0.1 μmole (U-^{14}C)-TdR or 20.1 μCi/0.4 μmole ^{3}H-uridine was injected through the cannula 15 min before sacrifice and removal of tissues. GZ in tissues was determined in the neutralized, PCA-soluble extracts as described in the text and expressed as μg per g frozen weight of tissues. Specific incorporations of TdR and UR into DNA were determined in the PCA-insoluble pellet and the results are expressed as percent of the corresponding saline-treated controls. The average saline-treated control values for TdR incorporation into DNA (dpm/mg DNA) were 23, 100; 22, 500 and 23, 800; and for UR incorporation into DNA (dpm/mg DNA) were 8, 460; 7, 690 and 9, 210 in the cases of spleen from normal, L1210-bearing and L1210/GZ-bearing mice respectively. The individual control values ranged between 93 and 107% of their means. Each number is an average of two experimental values ±0.5 range.

was, however, detected. Both non-leukemic and leukemic spleens showed a similar ability to incorporate uridine or thymidine into RNA and DNA (see legend of Table 4).

As shown in Table 4 and consistent with the data on GZ uptake in L1210 and L1210/GZ cells, the inhibition of incorporation of thymidine was higher in "L1210 spleen" than in "L1210/GZ spleen". In normal spleen cells, the inhibition was intermediate. Incorporation of uridine into DNA was similarly affected by the drug although the inhibition was less marked than that of thymidine into DNA.

Ribonucleotide profile of normal and leukemic spleen

A typical column chromatographic separation of the free ribonucleoside mono, di- and triphosphates found in normal and leukemic spleens is shown in Fig. 2. Figure 2(A) represents the ribonucleotide profile of normal mouse spleens (average 67 mg each). In this chromatograph IMP is the major component while ATP as well as other triphosphate pools were present at a considerably lower level. This chromatograph was reproduced in 6 separate experiments using the DBA/2J mice. The results of other experiments, not reported here, indicated that the NTP pools vary depending on the strain of mice. For example, high ATP pools were isolated from normal spleens obtained from Swiss and C57Bl/6J mice. Figures 2B and 2D represent the chromatographic separation of the ribonucleoside mono, di- and triphosphates found in leukemic spleen at 2 and 4 days, respectively after an i.v. administration of 10^6 L1210 cells. These chromatographs show a progressive increase in the ATP pool with time after L1210 inoculation. The increase was in parallel with the increase in spleen weight (160–240 mg), indicating progressive infiltration of the spleen by L1210 cells. Figure 2C shows the ribonucleotide profile of L1210 cells obtained from the peritoneal cavity of mice, which is similar to those obtained from L1210 containing spleens (Figs. 2B and SD).

The effect of continuous 48-hr infusion of GZ (30 mg/ml, 0.3 ml/hr) was investigated. The results show that treatment with GZ reduced the average spleen weight almost to control levels. Histopathological examination revealed the absence of leukemic cells in the spleen from drug-treated mice. Although under this schedule, GZ infusion did not prolong survival of leukemia bearing animals, the ribonucleotide profile returned to the non-leukemic pattern.

DISCUSSION

Administration of GZ by continuous i.v. infusion resulted in a substantial inhibition of incorporation of uridine and thymidine into DNA, particularly of hemopoietic tissues. The inhibition was quite marked within 1 hr even at concentrations of GZ in the infusion fluid as low as 5 mg/ml and when only a 1.5 mg of GZ was administered. In contrast, a single i.v. push of 1.5 mg GZ has no such effect on precursor incorporation. This demonstrates the usefulness of this infusion system for studying agents of very short half-life in unrestricted mice. The in-

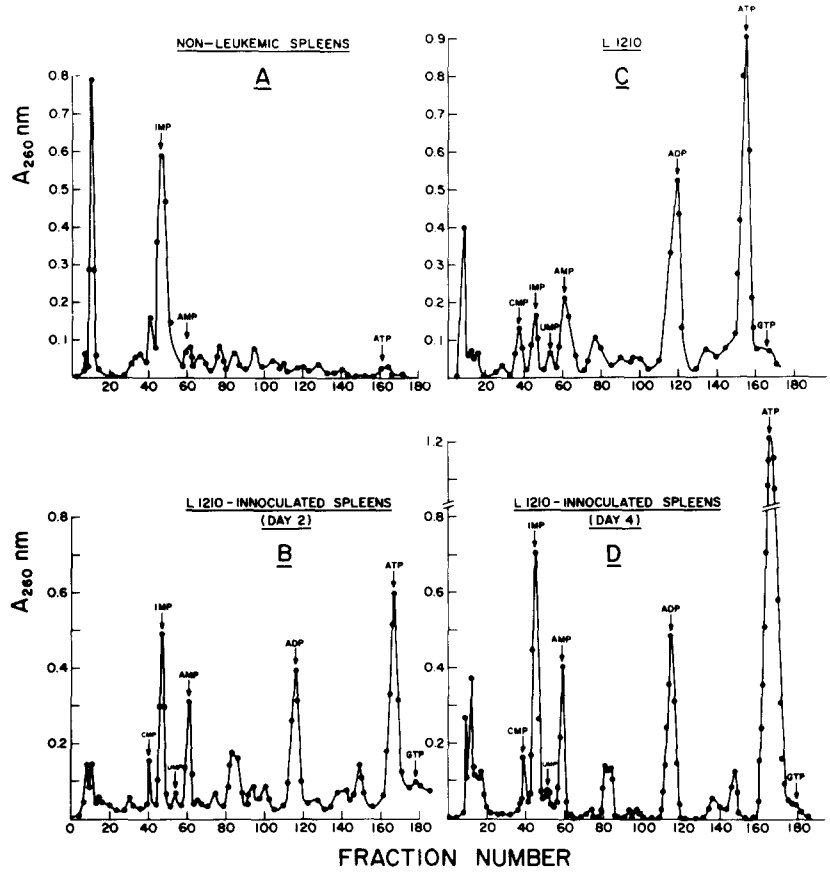

Fig. 2. DEAE-cellulose column chromatographic separation of free ribonucleotides found in normal DBA/2J mouse spleen (A) in leukemic spleen 2 days (B) and 4 days (D) after i.v. administration of 10^6 *L1210 cells. Ribonucleotide profile of ascites form of L1210 is shown in (C). Each chromatograph in A, B and D represents pooled extract of 4 spleens from which 20* A_{260} *absorbance units were absorbed on each column and eluted with an ammonium formate gradient* pH 4.4 (3 mM–0.3 M). *Similarly, 20* A_{260} *absorbance units were also used in the case of extract from ascitic L1210 cells.*

hibition of uridine incorporation into RNA and DNA of various tissues is consistent with the inhibition of ribonucleotide reductase by GZ as a primary mechanism of drug action [7]. The observed lack of inhibition of uptake of precursors or of the DNA polymerase reaction (C. Dave, unpublished data), are consistent with the contention that the reductase is probably the major site of drug action. The basis for the inhibition of incorporation of thymidine found in this study is not clear. Such an inhibition could occur due to possible changes in the pool sizes of deoxynucleotides, such as increase in dTTP or decreases in purine dNTP's. Alternately, it may represent another site of action of GZ.

Correlation of the inhibition of precursor incorporation with concentration of GZ in various tissues suggest that the differential sensitivity of tissues to the drug may be primarily related to drug uptake rather than to differential sensitivities of cellular ribonucleotide reductase(s). The transport data of GZ in L1210 cells suggest that the drug uptake occurs by a passive diffusion process. A decrease in uptake rate in L1210/GZ may therefore be related to plasma membrane changes in the resistant line. Indeed, enhanced antigenicity indicating possible membrane changes has been found to occur in L1210/GZ [18]. Similar membrane changes have also been postulated to occur in the case of L1210 cells resistant to 4,4′-diacetyldiphenyldiphenylurea-bis(guanylhydrazone)—a drug known to be taken up by a passive diffusion process [10, 19]. Thus membranes of different tissues may offer a different degree of resistance to entry of GZ and this may be the basis for its tissue-specificity.

It should, however, be pointed out that in a Chinese hamster ovary cell line, resistant to hydroxyurea, another ribonucleotide reductase inhibitor, the occurrence of an isoenzyme was found to be the basis for drug resistance [20]. Thus, in analogy with hydroxyurea, both altered

uptake of GZ and altered sensitivity of the reductase to the drug could be determinant of sensitivity to GZ. Based upon this premise, the determination of uptake of GZ and of incorporation of uridine into DNA would possibly indicate the likelihood that target cells are sensitive to GZ. Peripheral or bone marrow lymphoblasts from leukemic patients may be considered as potential target cells in this respect.

Low concentrations of triphosphate pools in normal spleen may be a unique occurrence in these mice since the corresponding profiles in two other strains of mice was akin to that seen in liver [14, 15]. Since we are determining steady-state concentrations of nucleotide pools the low concentrations may represent very rapid turnover. Alternately, these may represent high activity of phosphates in normal DBA/2J spleens. The data obtained on the ribonucleotide pools of spleen in non-leukemic versus leukemic mice demonstrate that qualitative and quantitative differences exist in spleen as a result of infiltration by leukemic cells. After infusion with GZ, a dramatic decrease occurred in the leukemic spleen size and also in the profile of ribonucleotides which reverted to the non-leukemic pattern. Such differences in the pool sizes may be indicative of differences existing in the regulation and biosynthetic needs of the cell types involved. Whether such differences could be exploited to diagnose potential or existing malignancy or to predict sensitivity towards a particular therapeutic regimen requires further investigation.

In conclusion, the results reported herein are in agreement with the earlier finding by Brockman *et al.* [7] indicating that the mechanism of action of guanazole is mediated through inhibition of ribonucleoside diphosphate reductase in different normal and tumor tissues in mice. The mechanism of selectivity of action, however, seems to be primarily dependent upon drug uptake.

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Biochemical and Karyological Properties of Cells Resistant to the Quinazoline Antifolate, Methasquin*

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Abstract—*Eleven methasquin-resistant sublines were selected* in vitro *from a Chinese hamster cell line and maintained at drug concentrations of* 0.01–50.0 μg/ml. *Dose–response data showed that increases in resistance ranged from* 6- *to* 6500-*fold and that all sublines were cross-resistant to methotrexate. Dihydrofolate reductase activity was elevated* 3- *to* 165-*fold in the* 11 *methasquin-resistant lines. Relationships between drug response and enzyme activity suggested that for* 6 *sublines increased dihydrofolate reductase may be the primary determinant of response, whereas additional factors such as quantitative change in other tetrahydrofolate enzymes may influence resistance to antifolate in the other* 5. *Karyotype analysis of conventionally stained metaphase cells revealed the presence of nonuniformly altered chromosomes. Strikingly long chromosome segments were found on* 1 *homologue of chromosome* 2 *or* 4 *as a consistent feature of most, but not all, of the* 11 *methasquin-resistant sublines. The similarity of these findings to those previously reported for methotrexate sublines and more recent results obtained by trypsin-Giemsa banding analysis provide evidence of specific chromosomal alteration associated with increased intracellular dihydrofolate reductase.*

INTRODUCTION

BIOLOGICAL and biochemical studies of a series of Chinese hamster sublines selected with and resistant to methotrexate (MTX) and cross-resistant to the quinazoline antifolate, methasquin (MQ), demonstrated that an increased amount of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$-oxidoreductase, EC 1.5.1.3) was a primary but not the only determinant of drug response in these cells [1]. Although dihydrofolate reductase activity was elevated in all of the 9 resistant sublines, the disproportionate relationships between enzyme activity and antifolate resistance, as well as the differential response of the various sublines to MTX and MQ, suggested more than one mode of resistance. Further, the resistant sublines could be classified into 2 groups, with respect to each antifolate, when degree of resistance was correlated with enzyme increase. Partial purification and characterization of dihydrofolate reductase from drug-sensitive parent cells and the 4 MTX-resistant sublines chosen for further investigation revealed the presence, in 2 of the 4 lines, of enzyme with decreased affinity for MTX but not for MQ [2]. Those cells which synthesized the novel enzyme were relatively more sensitive to the quinazoline antifolate than to MTX. The various observations prompted a similar study of a series of resistant sublines derived by direct exposure to MQ.

Prior chromosomal studies of MTX-resistant Chinese hamster lines suggested specificity of chromosome alteration associated with elevated levels of dihydrofolate reductase [1]. Furthermore, we earlier described specific karyotypic alteration of a marker chromosome in MTX-resistant sublines of mouse leukemia L1210 [3]. This report describes drug resistance patterns and dihydrofolate reductase activity of 11 Chinese hamster sublines selected by MQ and provides further evidence of specific cytogenetic abnormalities.

MATERIAL AND METHODS

The 11 MQ-resistant sublines included in this study were selected from mass cultures of parent

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DC-3F cells [4] by stepwise increases in concentration of MQ. Six sublines were derived independently of any other. Subline DC-3F/MQ8 (Table 1) was separated from DC-3F/MQ2 after 15 days of exposure to a low concentration of MQ. DC-3F/MQ19 was separated from DC-3F/MQ11, and DC-3F/MQ39 from DC-3F/MQ19, after drug exposure for 47 and 314 days, respectively. Eagle's minimum essential medium supplemented with 10% fetal calf serum was used throughout.

growth phase at time of enzyme assay. Protein was determined by the method of Lowry [7].

MQ was prepared in the laboratories of Dr. John Davoll and Dr. John Dice of Parke, Davis and Company, Ann Arbor, Mich. and supplied by Dr. Harry B. Wood of the Cancer Chemotherapy National Service Center. Its biological activities have been detailed [8]. MTX was obtained from Lederle Laboratories, Pearl River, N.Y. The 2 antifolates have similar mol. wt, 438.4 and 454.4, respectively.

Table 1. Concentration of methasquin at which resistant lines were maintained, response to methasquin and methotrexate in terms of ED_{50}, *and degree of resistance and cross-resistance*

Cell line	Maintenance concentration (μg/ml)	Methasquin ED_{50}* (μg/ml)	Methasquin Degree of resistance†	Methotrexate ED_{50}* (μg/ml)	Methotrexate Degree of cross-resistance	ED_{50} ratio‡
DC-3F		0.012	1	0.0067	1	1.8
DC-3F/MQ2	0.01	0.071	5.9	0.038	5.7	1.9
DC-3F/MQ11	0.1	0.23	19	0.17	25	1.4
DC-3F/MQ10	1.0	1.4	117	1.2	179	1.2
DC-3F/MQ20	1.0	1.6	133	1.6	239	1.0
DC-3F/MQ21	1.0	1.3	108	0.64	95	2.0
DC-3F/MQ25	1.0	1.1	92	1.1	164	1.0
DC-3F/MQ29	1.0	1.6	133	1.3	194	1.2
DC-3F/MQ31	1.0	1.6	133	1.7	254	0.9
DC-3F/MQ8	10.0	22	1833	45	6716	0.5
DC-3F/MQ19	10.0	19	1583	25	3731	0.8
DC-3F/MQ39	50.0	80	6667	185	27600	0.4

*ED_{50} value (50% inhibitory concentration) is based on 2–3 experimental determinations.
†Resistance is expressed as ratio of ED_{50} values for resistant to parental line; ratios were calculated as antilog of difference between log ED_{50} values obtained by computer.
‡ED_{50} of methasquin: ED_{50} of methotrexate, as calculated from molar equivalents of calculated values.

Response of resistant sublines to MQ and MTX was determined in a 3-day assay procedure based on relative cell counts in control and drug-treated cultures. Resistant cells were grown for 12–15 days in drug-free medium, with 2 transfers, prior to assay. Details of experiments and data analysis are essentially as previously described [1, 4], except that a thymidine supplement was not used. The dose effective in inhibiting and/or killing 50% of the cell population by the end of the 3-day period is reported as an ED_{50} value.

Dihydrofolate reductase activity was determined by previously described procedures [1,2,5]. Methods described earlier were also followed for measuring 5, 10-methylenetetrahydrofolate dehydrogenase activity either singly in reaction mixtures containing the formaldehyde adduct of dl,L-tetrahydrofolate as substrate, or coupled to serine hydroxymethyltransferase in reactions containing serine in place of formaldehyde [6]. Cells were grown for 13–14 days in absence of drug and were in exponential

Karyotype analysis and determination of chromosome numbers were made on conventional acetic orcein stained preparations, as for the MTX-resistant sublines [1]. In the present study, chromosomes were identified and classified with aid of recent results obtained by trypsin-Giemsa banding analysis [9, 10]. Modal chromosome number was derived from counts of 20–50 cells per subline. Ploidy estimates are based on surveys of 200 consecutive metaphase cells per line. Karyological determinations were carried out within 4–6 months after maintenance level of drug had been reached and were concurrent with drug response and enzyme activity determinations.

RESULTS

Resistance and cross-resistance

Response of drug-sensitive DC-3F cells and of each of the resistant sublines to both MQ and MTX is indicated by the ED_{50} values listed in Table 1. There was close correspondence be-

tween selective concentration of MQ and response to that agent for all resistant lines. Further, all sublines were similarly cross-resistant to MTX, as reflected in the calculated ED_{50} ratios (Table 1).

Correlation of dihydrofolate reductase activity with response to antifolates

All sublines had more dihydrofolate reductase activity than DC-3F (Table 2). Sublines with the greatest degree of resistance, viz. DC-3F/MQ8, DC-3F/MQ19 and DC-3F/MQ39, had the highest enzyme activity levels. However, when enzyme activity values are plotted against ED_{50} values (Fig. 1), the 11 MQ selected sublines appear to fall into 2 groups differing in the relationship between enzyme level and drug response. The 5 sublines in Group 2 demonstrate comparatively higher levels of resistance in relation to dihydrofolate activity than do the 6 sublines and parental DC-3F cells in Group 1. All cell lines responded similarly to the 2 antifolates (Table 1). Therefore, differential sensitivity to MQ by the dihydrofolate reductases of each group of sublines, as previously observed for MTX-resistant lines [2], does not apparently account for the disparity between enzyme and resistance levels; a study of the inhibition by MTX and MQ of the dihydrofolate reductase activity of representative sublines DC-3F/MQ19 (Group 2) and DC-3F/MQ25 (Group 1) demonstrated similar drug-titration patterns (Fig. 2).

Table 2. Dihydrofolate reductase activity of drug-sensitive parent line and methasquin-resistant sublines. The value for DC-3F line ± S.D. is based on 5 independent enzyme assays and was previously reported [3]. All other values were obtained in 2–3 independent determinations

Cell line	Specific activity*	Degree of increase†
DC-3F	0.41 ± 0.5	1
DC-3F/MQ2	1.60	3.9
DC-3F/MQ11	5.27	13
DC-3F/MQ10	1.20	2.9
DC-3F/MQ20	19.85	48
DC-3F/MQ21	35.24	86
DC-3F/MQ25	52.65	128
DC-3F/MQ29	49.85	121
DC-3F/MQ31	1.60	3.9
DC-3F/MQ8	58.99	144
DC-3F/MQ19	62.09	151
DC-3F/MQ39	67.83	165

*μmole of dihydrofolate reduced/hr/mg protein.
†Ratio of specific activities of resistant to parent cell line.

Activity of other enzymes

Low levels of serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate dehydrogenase activity were detected in cell extracts of the parent line, DC-3F and 4 representative resistant sublines (Table 3). Both enzyme activities in the 2 sublines from Group 2 resembled those determined for DC-3F with the greatest difference, a 30% lower level of dehydrogenase, displayed by DC-3F/MQ19. In contrast, dehydrogenase levels, when assayed separately, were 50–60% lower for the resistant sublines of Group 1. When coupled to serine hydroxymethyltransferase, the dehydrogenase activity of DC-3F/MQ29 was similarly low. The combined serine hydroxymethyltransferase and dehydrogenase activity of DC-3F/MQ11, also in Group 1, was somewhat higher than that of DC-3F/MQ29, but was, nonetheless, lower than the parental value.

Chromosome studies

Seven of the 11 MQ-resistant sublines had chromosome numbers predominantly in the diploid range, while 3 sublines were near-tetraploid (Table 4). In the 11th subline, DC-3F/MQ29, the frequency of "tetraploid" metaphases was consistently 20–25%. Of the 3 independently-derived hypotetraploid lines, DC-3F/MQ10 and DC-3F/MQ31 had low levels of dihydrofolate reductase relative to degree of resistance (thereby placing them in Group 2), while DC-3F/MQ20 had considerably higher enzyme activity. The effect of tetraploidy *per se* on drug response and enzyme activity is not immediately apparent.

Karyotype analysis of the 8 "diploid" sublines revealed seemingly nonuniform chromosomal alterations similar to those previously observed in MTX-resistant cells [1]. As before, unusually long chromosome segments consistently were found on the long of arms of chromosomes 2 and 4, particularly. Representative karyotypes are presented (Fig. 3) for parent DC-3F cells and 3 of the 5 MQ-resistant sublines with very high dihydrofolate reductase activity (Table 2). The abnormal chromosome arms are strikingly longer than those of any of the normal chromosomes of the Chinese hamster. DC-3F/MQ39 was derived from DC-3F/MQ19 and is karyotypically similar to it. The only other MQ-resistant subline with a greater than 100-fold increase in enzyme activity is DC-3F/MQ25 (Table 2). It was found to have an abnormally long chromosome 9, a less frequent alteration observed also in MTX-resistant cells [1]. In these MQ-resistant sublines the only other consistent

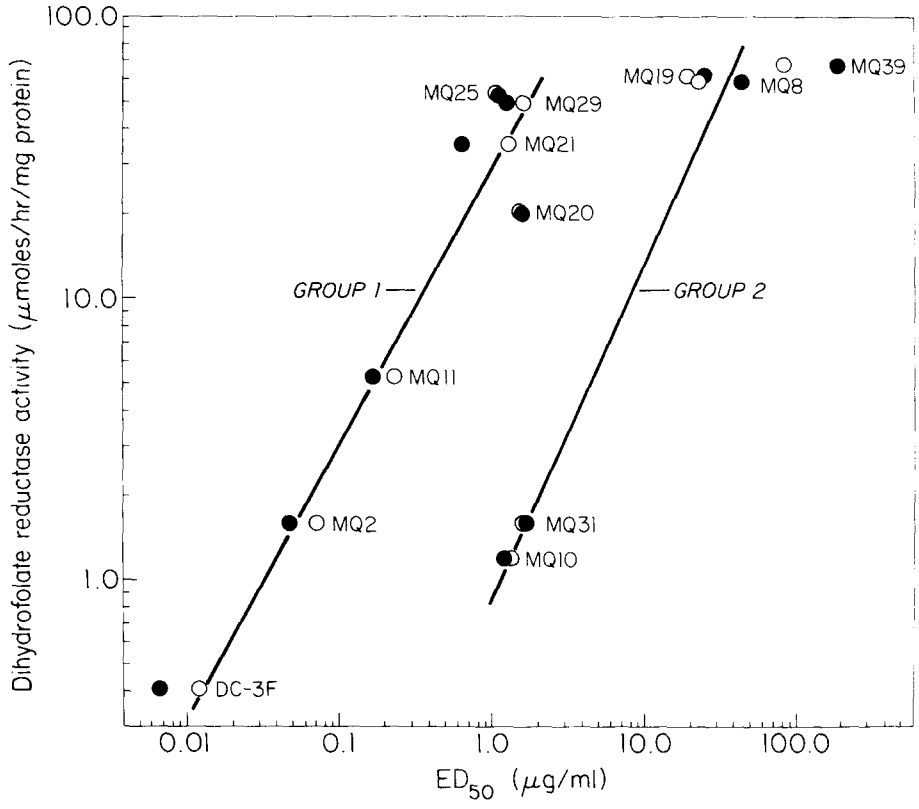

Fig. 1. Relationship between dihydrofolate reductase activity and response to MQ and MTX in terms of ED_{50} *for drug-sensitive DC-3F line and MQ-resistant sublines. Sublines are designated by abbreviated form of line name (Table 1). Methasquin response, ○; methotrexate response ●.*

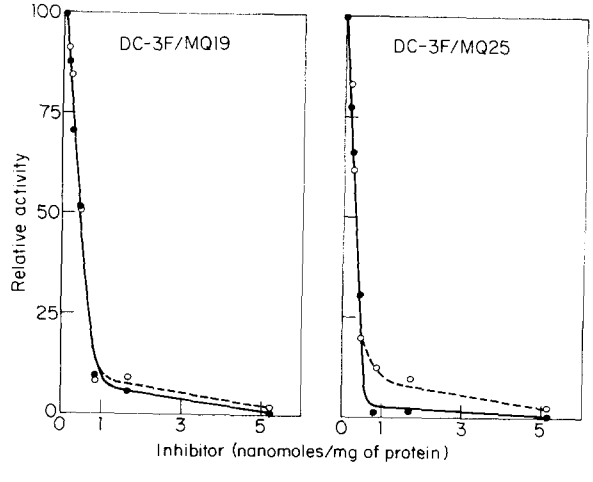

Fig. 2. Inhibition of the reduction of dihydrofolate by MTX (●) and MQ (○). Enzyme activity was determined following preincubation of cell extracts with different amounts of the inhibitors at pH 6.5. Reaction mixtures contained equivalent amounts of dihydrofolate reductase activity of either DC-3F/MQ19 or DC-3F/MQ25. Inhibitor is described in terms of the amount used/mg of protein and enzyme activity is expressed relative to uninhibited activity taken as 100.

karyotypic abnormalities characterized parent DC-3F cells as well: long arm translocation of chromosome 5, short arm translocation of chromosome 6, long arm deletion of chromosome 7 and missing or deleted X chromosomes.

Another cytologic feature of MQ-resistant cells which also characterized cells selected by MTX was the unstable configuration of a No. 2 chromosome [1]. During the course of development of resistance, examination of metaphase cells of sublines DC-3F/MQ8 and DC-3F/MQ11 revealed that usually one chromosome 2 showed either a deletion, a break, or, more commonly, a translocation to it of another centromere-containing chromosome or chromosomal segment (Fig. 4). Thus, the structural abnormality was of several different types and involved several chromosomes in addition to the No. 2. Usually only one of the homologues and only one arm of a chromosome 2 appeared affected. It is obvious even from conventionally stained chromosomes (Fig. 4) that the presumed

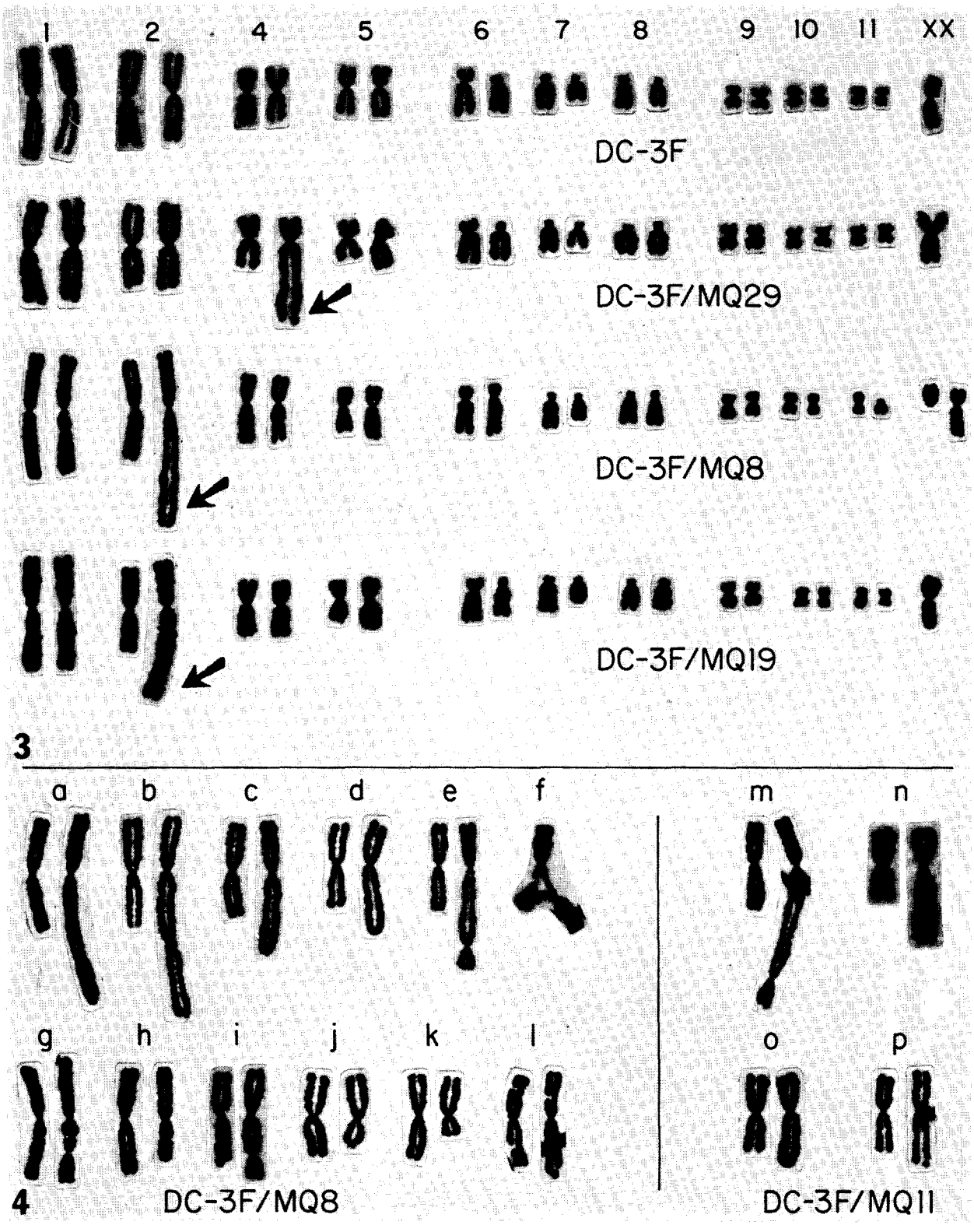

Fig. 3. Karyotypes of parent DC-3F line and 3 methasquin-resistant sublines with greater than 100-fold increases in dihydrofolate reductase activity. Abnormally long chromosome segments are indicated by arrows.

Fig. 4. Sets of No. 2 chromosomes from metaphase cells of sublines DC-3F/MQ8 and DC-3F/MQ11 karyotyped during course of development of resistance. Normal chromosome 2 is at left, for each pair. Note translocated acrocentric chromosomes with distal centromeres (sets a, c, d, h, j, o). The abnormal No. 2 of Set a has a long segment in addition to the distally translocated acrocentric, while the abnormal No. 2 of Sets h and j show partial deletions of the abnormal arm. Some abnormal No. 2 chromosomes had strikingly long arms apparently without an additional centromere (Sets b, n, p). In some cells a probable No. 5 chromosome was translocated to a No. 2 (Sets e, m and possibly g and i). Sometimes a chromosome 2 was deleted without an apparent translocation (Set k). Chromosome breaks or gaps were observed frequently (Sets f, l and p). Set f is a quadriradial resulting from a reciprocal translocation between both No. 2 chromosomes.

Table 3. Enzymic activity of methasquin-resistant Chinese hamster cell lines to form and interconvert tetrahydrofolate coenzymes

Cell line	Group*	Methylenetetrahydrofolate dehydrogenase activity†	
		Assayed separately‡	Coupled to serine hydroxymethyltransferase§
DC-3F	1	2.6	5.4
DC-3F/MQ19	2	1.8	4.6
DC-3F/MQ10	2	2.8	6.0
DC-3F/MQ29	1	1.0	1.9
DC-3F/MQ11	1	1.2	3.5

**See* Chart 1.
†Expressed as μmole of 5,10-methenyltetrahydrofolate formed per hr per mg of protein.
‡Assay with 5,10-methylenetetrahydrofolate (formaldehyde adduct of tetrahydrofolate) as substrate.
§Activity determined in reaction mixture containing tetrahydrofolate and serine as C-1 donor.

Table 4. Chromosome characteristics of parent cell line and drug-resistant sublines. Modal chromosome numbers are based on counts of 20–50 *metaphase cells per line. Cells with chromosome numbers in the diploid range* (20–25 *chromosomes*) *were scored as "diploid" in a survey of at least* 200 *consecutive metaphases per cell line*

Cell line	Maintenance concentration of methasquin (μg/ml)	Modal chromosome number	Diploid cells (%)
DC-3F	0	21	97.5
DC-3F/MQ2	0.01	21,22	99.0
DC-3F/MQ11	0.1	21	99.0
DC-3F/MQ10	1.0	42	0.0
DC-3F/MQ20	1.0	39,40	0.0
DC-3F/MQ21	1.0	22	97.0
DC-3F/MQ25	1.0	21	94.5
DC-3F/MQ29	1.0	21	79.5
DC-3F/MQ31	1.0	39,40	0.0
DC-3F/MQ8	10.0	22	94.5
DC-3F/MQ19	10.0	21	94.5
DC-3F/MQ39	50.0	21	97.5

breakpoint in the No. 2 was not always at the same site, in whichever arm was involved. By the time that the sublines were fully resistant (i.e. no evidence of toxicity) to their final selective concentration, cells were karyotypically stable, and the configuration of the No. 2 or other altered chromosome was consistent (Fig. 3).

DISCUSSION

All of the MQ-resistant sublines had elevated levels of dihydrofolate reductase even when selected with very low concentrations of antifolate (0.01 and 0.1 μg/ml), thus implicating this enzyme activity as a primary determinant of drug response in this system. Moreover, for the 6 MQ-resistant sublines of Group 1, there is close correspondence between degree of increase in target enzyme activity and resistance (Tables 1 and 2). As previously discerned for resistant sublines directly selected with MTX [1], an additional factor appears operative for some of the antifolate-resistant populations; the 5 sublines of Group 2 are considerably more resistant than would be expected solely on the basis of enzyme activity (Fig. 1). Two highly resistant Group 2 sublines, DC-3F/MQ8 and DC-3F/MQ19, derived from the less resistant Group 1 sublines, DC-3F/MQ2 and DC-3F/MQ11, sometime after the selection process was begun (see Material and Methods) apparently developed greater resistance relative to target enzyme activity as a result of higher selection pressure (Fig. 1). This observation suggests that

an additional contributory determinant is expressed at high selective concentrations of antifolate.

In the earlier studies of Chinese hamster cells selected with MTX [2], we found, in several of the sublines, a dihydrofolate reductase structurally different from parent DC-3F type. In drug titration studies, the altered enzyme showed a decreased affinity for MTX but an unchanged sensitivity to MQ. The presence of the mutant enzyme form was reflected in the observed differential response of the resistant cells to the two antifolates; sublines with altered enzyme were relatively more resistant to MTX than to the quinazoline analogue. All of the MQ-resistant sublines, however, responded similarly to the two drugs. Furthermore, kinetic analysis of the inhibition by the two antifolates of the enzymic reduction of dihydrofolate demonstrated similar drug titration patterns. Therefore, the greater resistance of Group 2 sublines does not appear to be the result of an altered dihydrofolate reductase similar to that detected and characterized in MTX-resistant cells.

Nevertheless, two clearly distinguishable groups of sublines differ in the relationship of resistance level to level of dihydrofolate reductase activity. Several explanations are possible. Sublines of Group 2 (Fig. 1), with relatively high resistance in relation to enzyme level, might synthesize a dihydrofolate reductase that is structurally different from that in Group 1 sublines and from the mutant enzyme form found in MTX-selected cells [2]. Such an alteration could influence the kinetics of intracellular enzyme activity in the presence of drug. Alternatively, other enzymes of folate metabolism [11] may quantitatively or qualitatively differ in the two subline groups. As suggested by the values for the serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate dehydrogenase activities of selected sublines of Groups 1 and 2 (Table 3), decreased levels of enzymes needed for efficient utilization of tetrahydrofolate, below the parental cell type level, might prevent the full expression of increased dihydrofolate reductase activity. In addition to enzyme differences, dissimilarities in active transport of MQ could contribute to the basis for the two groups.

Cytogenetic analysis of MQ-resistant cells strengthened previous observations of specific chromosome abnormalities in antifolate-resistant cells with increased levels of dihydrofolate reductase [1, 3, 12, 13]. In drug-resistant Chinese hamster cells the abnormalities are manifested as strikingly long chromosome segments most frequently marking chromosomes 2 and 4. Recently we extended these observations by examination of 13 of the "diploid" sublines independently selected with either MTX or MQ by means of trypsin-Giemsa banding methods [9, 10]. As briefly described elsewhere [14, 15], in those 7 sublines with greater than 100-fold increases in dihydrofolate reductase activity, a long, homogeneously staining region ("HSR") was consistently present, usually on the long arm of a chromosome 2 or otherwise on a chromosome 4 or 9 and, in one instance, on an unidentified marker chromosome. Further evidence of the relationship between the anomalous chromosome region and resistance to MTX or MQ associated with increased target enzyme activity was provided by another preliminary investigation [16]. When highly resistant, cloned cell populations were maintained in drug-free medium, there was a parallel decrease in antifolate resistance and average HSR length. In addition, we have found that in cell lines with high target enzyme activity as much as 2% of the total protein is dihydrofolate reductase [2], while in parent DC-3F cells the enzyme comprises approximately 0.012% of cell protein (A. M. Albrecht, unpublished observation). It thus appears that the strikingly long (HSR) chromosome segments are associated with overproduction of target enzyme. We are currently studying this phenomenon in an attempt to elucidate the functional significance of the anomalous chromosome region. Detailed karyotype analysis by chromosome banding techniques of the antifolate-resistant Chinese hamster sublines will be reported.

Comparisons of these MQ-selected sublines and the previously described MTX-selected sublines [2] reveal both similarities and dissimilarities. The apparently specific chromosome abnormalities are qualitatively similar. All of the 17 antifolate-resistant sublines derived from DC-3F exhibited elevated dihydrofolate reductase activity. Nevertheless, there was indication of an additional, though probably differing, determinant(s) of resistance for some sublines in each drug category. Increases in resistance and cross-resistance to MQ, as compared to MTX, were consistently of lesser magnitude. Should these various differences be reflected at the clinical level, MQ would be a useful adjunctive chemotherapeutic agent to MTX.

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Geographic and Time Trends of Coffee Imports and Bladder Cancer*

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Abstract—*Bladder cancer incidence rates were related to* per capita *coffee imports for ten countries and time trends in these variables were analyzed for the United States and Denmark. Among the ten countries, there was a weak positive association between coffee and bladder cancer for both men and women. There was a weak positive correlation of the time trends of coffee imports and bladder cancer incidence for men, but not women, in the United States. The trend of coffee imports for Denmark was dissimilar to both male and female trends in bladder cancer incidence. Because the relationships observed are neither strong nor consistent in direction, these results provide little support for an association of coffee drinking with the development of bladder cancer.*

INTRODUCTION

THE RELATIONSHIP between coffee drinking and development of bladder cancer has been the subject of several case-control studies [1–5]. These studies suggest a positive association [6]. Stocks [7] and Takahashi [8] reported that national rates of coffee importation were not related to bladder cancer mortality rates. However, an association between coffee consumption and incidence of bladder cancer might not be clearly reflected in mortality rates. Therefore geographic and temporal correlations of coffee consumption with incidence rate of bladder cancer were examined.

MATERIAL AND METHODS

This analysis was based on countries for which coffee and cigarette consumption figures and bladder cancer incidence rates were available. "The World Coffee Economy" presents national data on coffee imports for 1929 through 1960 [9]. National data on cigarette consumption also have been published [10]. The consumption figures in the present report are averages of annual data related to the population age 15 or greater. Incidence rates age-standardized to the European population were obtained from "Cancer Incidence in Five Continents" [11]. The rates for Canada and the United Kingdom are means of rates from the individual registries. Incidence rates for the "European" population were used for New Zealand. Data for South Africa have not been included as coffee and cigarette consumption were not linked with the specific racial groups for which incidence rates were published. For Denmark and Connecticut, incidence rates have been measured over extended periods [12–14], permitting study of time trends.

Trends presented in this report are based on age-standardized annual incidence rates per 100,000. However, trends in rates for men and women age 65–74 also were examined. In all regression analyses, the two independent variables were the measures of coffee and cigarette consumption. The latter was included because cigarette smoking is a well established risk indicator for bladder cancer [15], and ignoring cigarette consumption might lead to the appearance of erroneous relationships between coffee and bladder cancer. Thus, regression coefficients presented for coffee imports have been adjusted for the effect of cigarette consumption. In each analysis the independent variable was the annual incidence rate.

RESULTS

The basic information for the international comparisons is presented in Table 1. Exposure data were taken from the period about 25 yr before the incidence data were collected in order to allow for the probable long induction period of bladder cancer [1]. The rate of coffee

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Table 1. Coffee and cigarette consumption and age-standardized bladder cancer incidence rates for 10 countries

	Coffee† (kg imported per adult per yr) 1935–1939	Cigarettes (number per adult per yr) 1935–1939	Bladder cancer (annual incidence per 100,000)‡ Male	Female
Japan (Miyagi)*	0.11	926	7.0	2.3
Norway	8.31	354	9.4	3.4
Germany (Democratic Rep.)*	3.12	786	9.5	2.0
Finland	8.37	1460	11.3	2.5
Sweden	10.16	380	14.5	5.2
Canada	2.34	810	17.9	5.2
Denmark	10.91	532	18.2	5.7
New Zealand	0.19	672	18.5	3.7
United Kingdom	0.45	1784	18.5	4.6
U.S. (Connecticut)*	8.69	1650	29.9	8.8

*Registration area.
†Regression coefficient (standard error). Male 0.45 (0.50). Female 0.23 (0.15).
‡Rates are for various periods from 1958 to 1966.

Table 2. Coffee and cigarette consumption and age-standardized bladder cancer incidence rates by time period, United States

	Coffee (kg imported per adult per yr)	Cigarettes (number per adult per yr)	Bladder cancer (annual incidence per 100,000)‖ Male	Female
1929	7.79	1390	—	—
1930–1934	8.00	1284	—	—
1935–1939	8.69	1650	10.6	4.8
1940–1944	9.65	2206	11.4	4.2
1945–1949	11.47	3044	15.5	5.3
1950–1954	10.24	3344	17.2	5.3
1955–1959	10.44	3496	20.3	5.6
1960–1964	10.71§	3854	20.3	6.2
1965–1968	——	3808	23.4	6.4

§For 1960.
‖For Connecticut.

importation has a range of about 100-fold, from 0.11 in Japan to 10.91 in Denmark. Regular coffee-bladder cancer trends are not apparent, but some of this irregularity seems attributable to differences in cigarette consumption. In two countries with low or moderate incidence rates but relatively high coffee consumption—Norway and Sweden—cigarette consumption was quite low. In the United Kingdom, where bladder cancer rates were high but there was little coffee consumption, the annual number of cigarettes smoked per adult was high.

The age-adjusted annual bladder cancer incidence rate for men was estimated to increase 0.45 per 100,000 for each kilogram of coffee imported per person per year. The corresponding rate of increase for women was estimated as 0.23. Two-sided *P*-values for these regression coefficients are 0.59 and 0.17, respectively. However, a *protective* effect of coffee drinking on bladder cancer seems most unlikely, judging from previous studies, so one-sided *P*-values of 0.30 and 0.08 might be more appropriate.

Data on time trends in the U.S. and Denmark are presented in Tables 2 and 3. In both countries, bladder cancer rates for both men and women have increased, this increase being somewhat greater in Denmark than in the U.S. In Denmark, a uniform trend in coffee imports is not apparent; there was a marked decrease around the time of World War II. There has been a slow increase in coffee imports in the U.S. In each area, cigarette consumption has risen sharply during the period of observation, the increase from the earliest time period to the most recent being about threefold.

Table 3. Coffee and cigarette consumption and age-standardized bladder cancer incidence rates by time period, Denmark

	Coffee (kg imported per adult per yr)	Cigarettes (number per adult per yr)	Bladder cancer (annual incidence per 100,000)	
			Male	Female
1929–1932	10.44	470	—	—
1933–1937	9.72	476	—	—
1938–1942	5.73	570	—	—
1943–1947	1.95	530	8.7	3.4
1948–1952	5.15	1116	11.9	3.9
1953–1957	8.76	1196	16.1	5.2
1958–1962	11.61*	1430	18.7	5.8
1963–1967	—	1522	23.8	6.4

*For 1958–1960.

Regression coefficients were computed for descriptive purposes, although the coefficients were based on only six observations for the U.S. and five for Denmark. Incidence rates for time periods beginning in the 1940s and after were related to exposure data for the period 10 yr earlier. There was a weak positive association of coffee imports with the bladder cancer rate for U.S. men (regression coefficient, 0.31) but not for U.S. women (regression coefficient, −0.19). In Denmark, the trend in coffee imports was dissimilar to the trends of both male and female rates of bladder cancer. The regression coefficients were −0.51 for men and −0.15 for women.

The geographic and temporal relationships of coffee imports to incidence rates for men and women age 65–74 were similar to the associations presented for the age-standardized rates.

DISCUSSION

Observations from case-control studies have not been consistent, but have suggested a positive association between coffee drinking and risk of bladder cancer. Cole reported relative risks of 1.3 in men and 2.5 in women for drinkers of one or more cups per day, compared to those who drank less [1]. Positive associations were found by Fraumeni *et al.* [2] in three of four sex-race groups, and Bross and Tidings [3] in five of six age-sex groups, although the over-all association was weakly negative for women in the latter study. A positive association also was observed in a study limited to women that was reported by Simon *et al.* [4]. However, dose–response relationships have not been observed in these studies and bladder cancer did not appear associated with coffee drinking in a study done in Canada [5].

Bladder cancer mortality rates have not been found to be correlated with rates of coffee importation [7, 8]. In the mortality studies the effects of cigarette smoking and induction period were not taken into account, but these factors were accommodated in the present analysis of incidence rates. Positive associations of coffee and bladder cancer were observed for the international comparisons, although these relationships are weak. The increasing bladder cancer rate for Connecticut men does follow the increase in U.S. coffee imports, but a similar trend was not observed for men in Denmark.

The bladder cancer rate increases for women in Connecticut and Denmark were less than those for men in the respective areas. These relatively small increases for women are consistent with the comparatively low exposure rates of women to the major known bladder cancer risk factors, cigarette smoking and occupation [6]. With tobacco consumption taken into account, there was no evidence that coffee contributed to the female rate increase in either area. Thus, on balance, the observed geographic and temporal trends provide little support for a coffee–bladder cancer association.

The irregularities in the results of the case-control studies suggest that observed positive associations of coffee and bladder cancer are not the result of a causal link [4]. The apparent lack of consistent geographic and temporal correlations also suggests this point of view. However, the present results have some important limitations. First, the analyses were, of necessity, based on only a small amount of information. Second, the measure of "exposure" is rather indirect; that is, the amount of coffee imported annually per adult over 15 may not be a good indicator of potentially relevant long-term coffee drinking patterns of individuals. Therefore, consistent changes of incidence rates in relation to coffee imports might not have been observed readily.

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Comparison of the Cytotoxicity of Methotrexate and Aminopterin on Methotrexate Resistant Murine Lymphoma Cells *in Vitro*

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Abstract—*The cytotoxicity of equivalent concentrations of methotrexate and aminopterin were compared on two methotrexate resistant sublines of murine lymphoma cells. At all concentrations of drug and for all durations of exposure the effects of the two agents were equivalent. Under the experimental conditions employed the superior transport properties of aminopterin failed to produce enhanced cytotoxicity as compared with methotrexate.*

INTRODUCTION

METHOTREXATE (MTX) superceded aminopterin in clinical use in the early 1950's and has remained to this date the most important and widely used folic acid antagonist in clinical cancer chemotherapy. Early toxicological studies in animals and in man indicated a somewhat superior therapeutic index for MTX, though for equivalent therapeutic effect it was suggested that the dose of MTX must be five to ten times higher than aminopterin. [1–3].

Recent studies by Goldman would suggest that at equivalent concentrations aminopterin is transported more efficiently into cells and hence on an equimolar basis may be a more effective cytotoxic agent than MTX [4]. With current protocols utilizing very high doses of MTX this biochemical advantage might be translated into both a better anti-tumour effect as well as a possible economic saving in terms of less drug being required. To test this hypothesis we compared the effect of very high concentrations of MTX and aminopterin on the viability of two lines of murine lymphoma cells, each relatively resistant to MTX, but by differing mechanisms. Under the experimental conditions employed we could discern no differences in effect between aminopterin and methotrexate when time and dose were varied.

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MATERIAL AND METHODS

Cells and cell culture

A subline of L5178Y lymphoma cells (L5178Y-MRT) which are capable of growing continuously in suspension culture at concentrations of methotrexate of 5×10^{-6}M was employed. The cells were grown in Fischer's medium for leukemic mouse cells (Grand Island Biologicals, New York, U.S.A.) supplemented with 10% foetal calf serum (Flow Laboratories Ltd., U.S.A.). Resistance in this cell line has been found previously to be related to impaired MTX transport [5, 6].

L1210 cells designated R3U (a gift from Dr. R. C. Jackson, Laboratory of Experimental Oncology, Indiana University) were grown in RPMI 1640 medium (Grand Island Biologicals, New York, U.S.A.) supplemented with 10% foetal calf serum. This cell line was capable of sustained growth at concentrations of methotrexate up to 10^{-6}M. Resistance in this cell line associated with an elevated level of dihydrofolate reductase, but normal MTX transport [7].

Cell viability assays were performed according to the method of Chu and Fischer [8] and with modifications previously described [9]. The method for the L1210 cells was essentially identical to that for the L5178Y cells. Under these conditions the cloning efficiency for the L5178Y cells was between 65–75% and for the L1210 cells between 60–70%. The data expressed in the survival curves represents the mean and standard deviation of the colony counts of five replicate cultures with each point expressed as a percentage of the non-treated

control cultures, which were given the value of 100%. Survival was determined under conditions where duration of exposure was kept constant at 24 hr and dose was progressively increased. In the time course survival curve the concentration of drug was set at 10^{-4}M and the survival at 6, 24 and 48 hr was measured.

Reagents

MTX was obtained as the sodium salt from Lederle Laboratories, Montreal, Canada. Aminopterin was obtained from Sigma Biochemicals, St. Louis, U.S.A.

RESULTS

Figure 1(a) and (b) shows the survival curves of the L5178Y and L1210 cells with percent

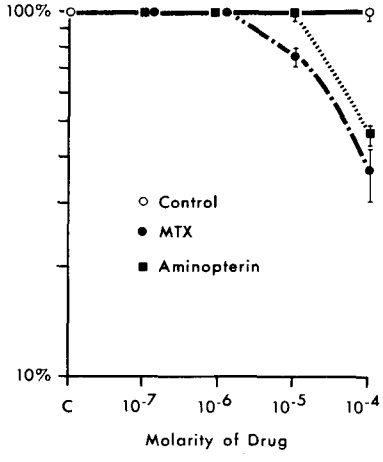

Fig. 1. (a) *Effect of increasing concentrations of methotrexate and aminopterin over a* 24 *hr period on the colony forming ability of L5178Y-MRT cells. Percent surviving fraction plotted against molarity of drug concentration.*

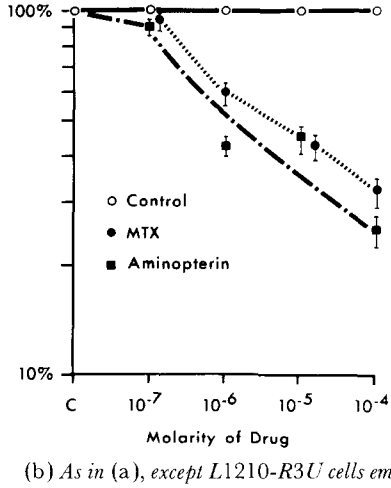

Fig. 1. (b) *As in* (a), *except L1210-R3U cells employed.*

surviving fraction plotted against increasing molarity of drug on a log-log scale. In neither circumstance was there any significant difference between the effect of aminopterin or MTX.

Figure 2(a) and (b) shows the effect of 10^{-4}M

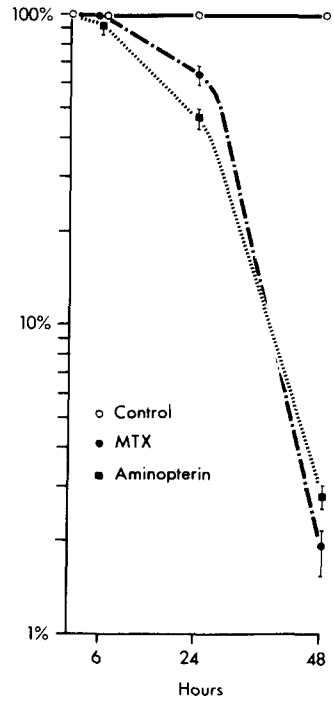

Fig. 2. (a) *Effect of* 6, 24, *and* 48 *hr exposure to* $10^{-4}M$ *methotrexate and aminopterin on the colony forming ability of L5178Y-MRT cells. Percent surviving fraction plotted against increasing duration of drug exposure.*

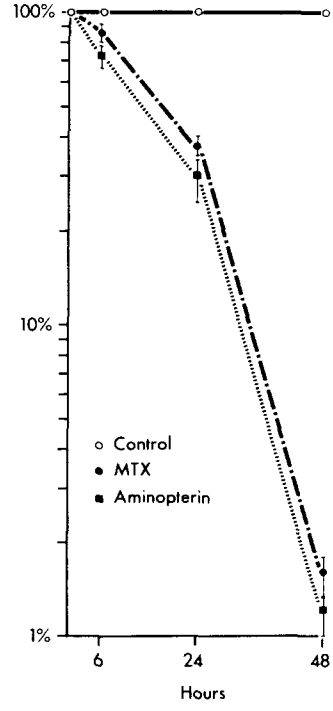

Fig. 2. (b) *As in* (a) *except L1210-R3U cells employed.*

drug concentration on the survival of the two cell lines with the duration of exposure extended to 48 hr. In both cell lines increasing exposure beyond 24 hr resulted in significant increase in fractional cell kill, but again no large differences between MTX and aminopterin were observed.

DISCUSSION

It is disappointing to observe that in our system aminopterin showed no advantage over methotrexate. The cell lines employed were deliberately selected because they were moderately resistant to methotrexate and by what appears to be the two principle mechanisms of resistance for mammalian cells [7]. No sweeping generalization from these negative results ought to be made. The superior transport properties of aminopterin noted by Goldman [4] may not be manifest in all tumour types or under all experimental conditions. Furthermore the pharmacokinetics of the two drugs in the patient may provide an advantage of one drug over the other which is not apparent in an *in vitro* system. In this connection, however, the pharmacokinetic differences between MTX and aminopterin that have been demonstrated in the mouse [10] seem to result in enhanced toxicity for aminopterin. The demonstration of a clear cut biological superiority of aminopterin over methotrexate in some tumour systems would lend encouragement for the search for human malignancies where the substitution of one antifolate for the other would offer significant advantages in terms of treatment.

CONCLUSION

In the two cell lines employed in these experiments each resistant to methotrexate by differing mechanisms, no evidence of superiority of aminopterin over methotrexate could be found in cell viability assays. Though there are theoretical considerations for believing aminopterin might be superior to methotrexate, this was not apparent in the *in vitro* conditions described in this report.

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Studies of the Growth of an Ascitic Tumour. I. Cellular and Subcellular Changes During the Tumour Growth

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Abstract—*The growth rate of the NK/Ly ascitic tumour decreases continuously but reversibly with the enlargement of the tumour mass. The increase in duration and distribution of cell cycle times is accompanied by structural alteration of nuclei and cell membranes, a reduced level of precursor incorporation into biopolymers and a decreased RNA polymerase activity. Besides the similarity in the nuclear proteins different types of non-histones are preferentially synthesized in the fast and slowly proliferating cell population. Furthermore, differences are observed in the decomposition rate of nuclear protein fractions. The more extensive binding of proteins to DNA is in harmony with these observations and all suggested that gene-expression decreases during tumour growth.*

INTRODUCTION

SINCE ascitic tumours are relatively homogeneous cell populations, they are widely used as test systems for experiments designed to obtain data on the characteristics and regulatory mechanisms of tumour growth. Numerous studies have provided evidences on the various changes both at the cellular and subcellular level during tumour growth [1–13] but very few were concerned with a comprehensive analysis of the same tumour. We have felt that detailed studies of the tumour growth using cell kinetic, biochemical and morphological approaches and concentrating mainly on the nuclear changes might help us not only to know more about the important steps involved in the steadily decreasing rate of cell proliferation but also to plan experiments on drug sensitivity at different stages of the tumour. The latter issue is based on several observations indicating the role of growth rate in the effectiveness of cancer chemotherapy [14–18].

MATERIAL AND METHODS

The NK/Ly ascitic tumor employed for these studies is a more or less diploid transplantable lymphoma [19] maintained in non-inbred CFLP mice by i.p. injection of 5×10^6 tumour cells weekly. The tumour has a tetraploid subline (5–10% of cells) and two marker chromosomes [20–22]. The animals were housed in plastic cages and fed with LATI pellet mouse diet and drinking water *ad libitum* (Laboratory Animals Breeding Center, LATI, Gödöllő, Hungary).

To determine growth curves, tumour-bearing mice were killed at various intervals after transplantation by cervical dislocation and the total number of ascitic tumour cells per mouse was counted in a haematocytometer. The doubling time of tumours was estimated from the computed growth curve fitting a Gompertzian equation to the experimental data.

In the labelling index experiments ^{3}H-thymidine (1 μCi/g body weight) was injected i.p. and the mice were killed 60 min later. For repeated labelling ^{3}H-thymidine was administered at 8 hr intervals. In the labelled-mitoses experiment, mice were killed at intervals of 2–4 hr after a single ^{3}H-thymidine injection. In each case the ascitic fluid was withdrawn with 0.6% sodium citrate in physiological saline. Smears were made on clean slides and fixed in methanol:glacial acid (3:1 v/v).

For preparation of autoradiographs, slides were dipped into an Ilford K5 emulsion, exposed for 1–2 weeks and developed in Microdol X. The number of labelled mitoses was determined on 250 mitoses and the data were analysed by the method of Steel and Hanes [23]. The fraction of labelled cells was counted from 1000 tumour cells.

The smears derived from single and repeated

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labelling experiments were used for cytophotometric studies. Feulgen staining was performed before autoradiography as follows: hydrolysis with 1 N hydrochloric acid at 60°C to ensure maximal dye binding and incubation for 1 hr in Schiff's reagent. Single cell DNA content was determined at 550 nm by Barr and Stroud microdensitometer type GN 2.

For electronmicroscopic study 0.25–0.25 ml of ascitic tumour was fixed in 2% OsO_4 according to the method of Palade [24]. In the case of double fixation the cells were fixed in phosphate buffered (pH 7.4) 2.5% glutaraldehyde and postfixed in OsO_4. For cytochemistry the tumour was fixed only with glutaraldehyde. The RNP "staining" was carried out according to Bernhard [25]. The ruthenium-red "staining" was performed in cacodylate buffered (pH 7.4) 2.5% glutaraldehyde for 1 hr. The fixative contained 500 μg/ml ruthenium red. The tumour cells were rinsed in sucrose (7.5%) containing cacodylate buffer and postfixed in ruthenium red containing OsO_4 for 3 hr in the dark. The specimens were dehydrated in a graded series of ethanol and propylene oxide solutions and embedded in Durcupan ACM. Ultrathin sections were stained for 30 min in saturated aqueous uranyl acetate and/or in lead citrate for 2 min [26]. Sections were examined with a JEM 7 A electron microscope.

For biochemical studies the tumour cells harvested at various times after the *in vivo* administration of labelled precursors were extracted twice with ice-cold 0.5 N perchloric acid (PCA). The pellet was treated with hot (70°C) 0.5 N PCA to release nucleic acids. The residue was solubilized in 0.1 N sodium hydroxide for protein measurements. The nucleotides were measured at 260 nm, while RNA, DNA and protein content were determined by the method described by Brown [27], Burton [28] and Lowry *et al.* [29] respectively.

For measuring RNA polymerase activity, nuclei were isolated in a hypotonic medium containing 0.3% Triton X-100 [30]. Magnesium and manganese activated RNA polymerase activity was assayed as described by Pogo [31].

Chromatin proteins were studied at 1, 6 and 24 hr after i.p. administration of 30 μCi ^{3}H-lysine and in some experiments of ^{3}H-arginine to 3–10 day-old ascitic tumours. In these experiments nuclei were purified according to Perry *et al.* [32]. Nuclear protein fractions were prepared by a sequential extraction procedure as follows: 0.15 M NaCl, 0.35 M NaCl, 0.2 N HCl and finally nuclear residual acidic proteins solubilized in 0.01 M sodium phosphate buffer (pH 6.8) containing 1% SDS. Proteins were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis as described by Webern and Osborne [33]. The gels stained with Coomassie-Brilliant-Blue were scanned in Joyce-Loebl "Chromoscan" densitometer at 575 nm, frozen at −20°C and mechanically sliced. The gel-slices were placed into scintillation vials, dissolved in 0.5 ml of 30% H_2O_2 at 50°C for 24 hr.

To estimate DNA-protein interactions, chromatin was isolated at low ionic strength [34] and binding of ethidium bromide to DNA was measured in a Farrand spectrofluorimeter at 335 nm excitation and at 593 nm fluorescent wavelength [35].

All radioactivity measurements were performed in a toluene-2-ethoxyethanol based scintillation fluid by using a Beckman LS-100 C spectrometer.

The following isotopes and fine chemicals were used: thymidine-6-^{3}H (s.a.*: 5 Ci/mM), uridine-5-^{3}H (s.a.: 5 Ci/mM), L-leucine-4,5-^{3}H (s.a.: 54 Ci/mM), L-arginine-5-^{3}H (s.a.: 16 Ci/mM), uridine-5-^{3}H-5'-triphosphate (s.a.: 1 Ci/mM). They were purchased from the Radiochemical Centre, Amersham. L-Lysine-6-^{3}H (s.a.: 291 mCi/mM) was purchased from the MTA Institute for Isotopes, Budapest. Ethidiumbromide (2,7-diamino-10-ethyl-9-phenylphenatridium) was produced by Calbiochem.

RESULTS

Cell kinetic studies

After transplantation of 5×10^6 NK/Ly ascitic tumour cells the growth rate decreased continuously. The experimental growth data were well fitted by a Gompertzian function (Fig. 1a). The proliferation rate of the tumour was influenced by the number of transplanted cells (Fig. 1b), but it was independent of tumour age (Fig. 1c). Among the main factors influencing the growth rate an increase of median cell cycle time was demonstrated with the labelled mitosis technique (Fig. 2, Table 1). In addition to this, the heterogeneity of the cell cycle time also increased as is indicated by the computed distributions (Fig. 3). The growth fraction at various stages of tumour growth was calculated from the data on the duration of cell cycle phases assuming an exponential distribution of cells in the cycle (Table 1). Repeated labelling experiments gave slightly higher values of growth

*Specific activity (s.a.).

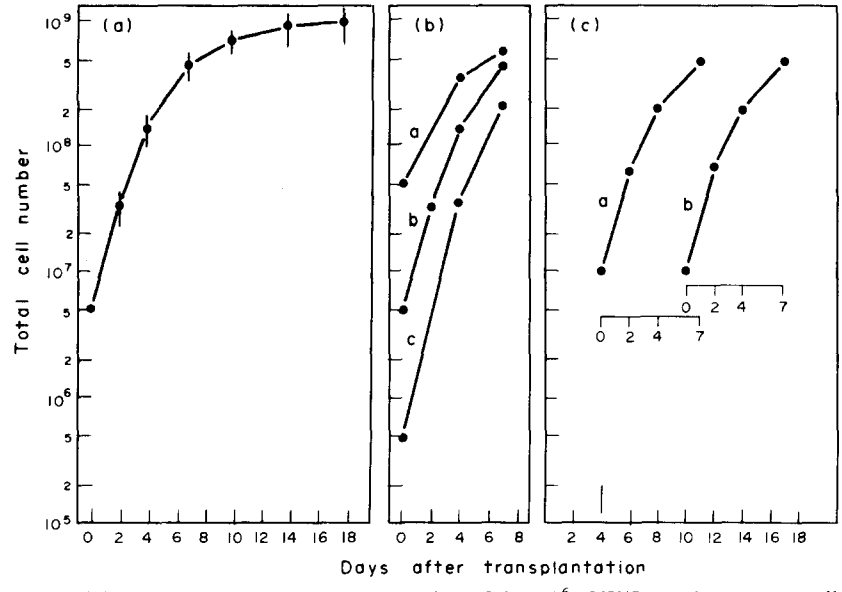

Fig. 1(a) *Growth curve after transplantation of* 5×10^6 *NK/Ly ascites tumour cells.* (b) *Growth curves after transplantation of* 5×10^7 (a), 5×10^6 (b) *and* 5×10^5 (c) *NK/Ly ascites tumour cells.* (c) *Growth curves after transplantation of* 10^7 *NK/Ly ascites tumour cells derived from* 4 *days* (a) *and* 10 *days* (b) *old tumours.*

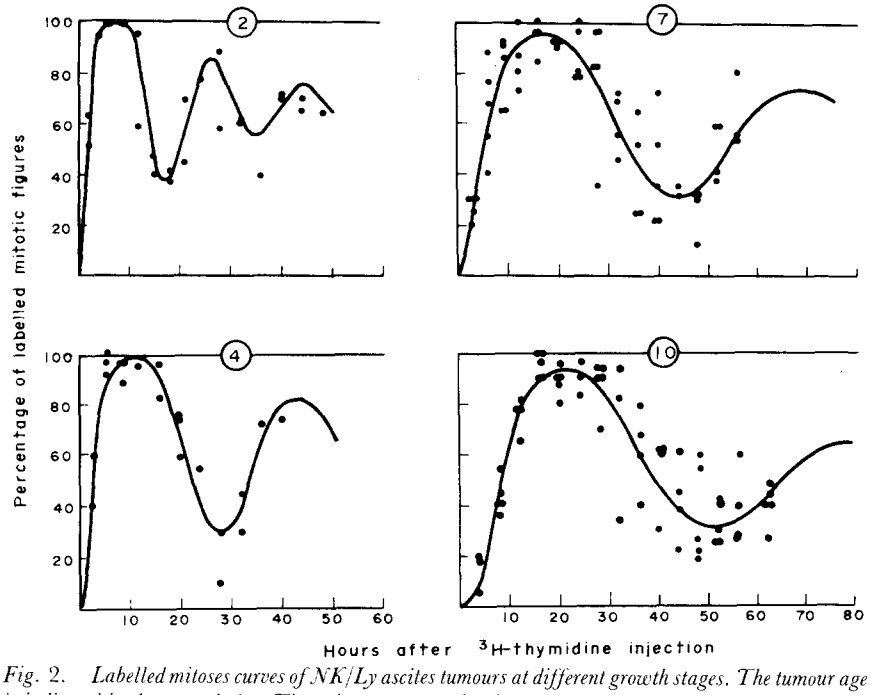

Fig. 2. *Labelled mitoses curves of NK/Ly ascites tumours at different growth stages. The tumour age is indicated in the open circles. The points represent the observed experimental values. The solid lines were computed from the experimental data according to the method of Steel and Hanes* [23].

fraction possibly because of the transition of cells from the proliferative to the non-proliferative stage and vice versa. Both the calculated and measured values of the growth fraction indicated a decreasing tendency in the ratio of proliferating cells (Fig. 4, Table 1). There was no appreciable change in cell loss factor with increasing tumour age up to 7 days (Table 1).

The cytophotometry was combined with autoradiography to establish the DNA content of the cells existing in the G_1 and G_2 phases. After a pulse ^{3}H-thymidine labelling the ratio of G_1 and G_2 cells was 71.5–27.5% in 7-day-old and 62.0–32.5% in 14-day-old tumours (Fig. 5a and b) indicating a slight increase of G_2 cells with the aging of the ascites tumour. Furthermore, the DNA content was measured in the non-labelled cells observed at the plateau of the labelling index in the repeated labelling experiment (i.e. non-proliferating cells) of 14-day-old

Table 1. Cell kinetic parameters at various growth stages of NK/Ly ascites tumour

Days after transplantation	2 days	4 days	7 days	10 days
Doubling time (hr)*	20.86	33.12	66.26	132.58
Median cell cycle time (hr)†	18.1	30.6	49.3	56.7
G_2 phase (hr)†	1.8	2.9	4.8	8.4
	(2.0;0.9)	(3.4;2.1)	(6.1;4.8)	(9.3;4.7)
S phase (hr)†	12.5	19.1	28.8	30.4
	(12.8;2.6)	(19.7;5.2)	(30.2;9.5)	(32.8;13.4)
G_1 phase (hr)†	3.0	8.3	14.7	16.4
	(4.0;3.4)	(8.3;0.6)	(14.9;2.0)	(17.0;4.6)
Labelling index (%)	64.8	55.0	42.5	36.5
	±3.4	±3.8	±6.3	±3.4
Growth fraction				
calculated	0.938	0.881	0.727	0.681
measured			0.82 -	0.67 -
			0.95	0.88
Mitosis index (‰)	23.7	8.7	8.5	10.4
	±6.7	±1.5	±4.2	±4.8
Cell loss factor	0.09	0.02	0.05	0.45

*Data derived from the computed growth curve.
†Data derived from the analysis of experimental values by method of Steel and Hanes [23]; median (mean; S.D.).

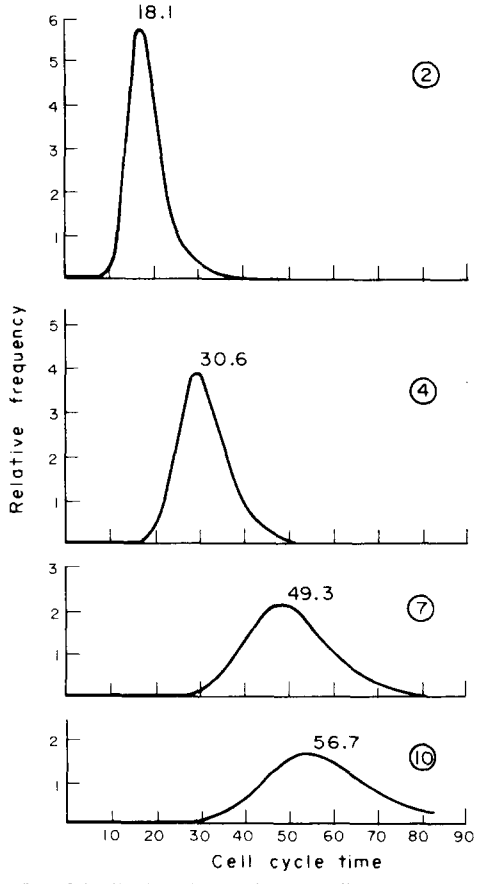

Fig. 3. Distribution of cell cycle time at different age (indicated in open circles) of NK/Ly ascites tumours. Above the curves the computed median cell cycle times are shown.

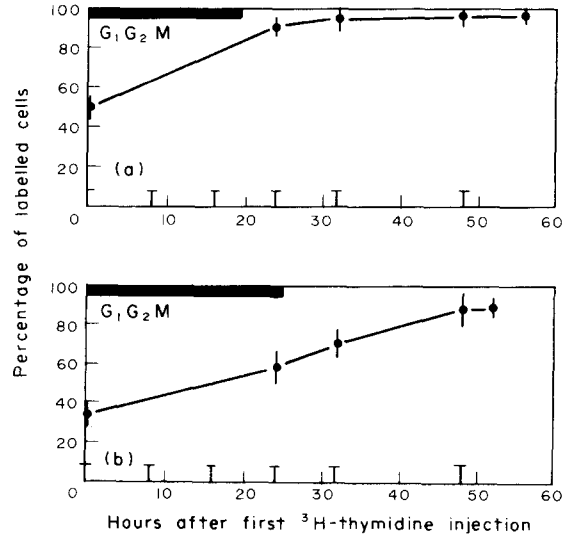

Fig. 4. Changes in the ratio of labelled NK/Ly ascites tumour cells after repeated ^{3}H-thymidine injections (T). (a—7-day-old, b—10-day-old tumour).

tumours. In this case the ratio of G_1 and G_2 cells was 75.0–19% (Fig. 5c).

Biochemical studies

Both the percentage of the labelled cells (Table 1) and the number of the silver grains observed above the nuclei decreased with the tumour age. These two parameters showed a change of DNA synthesis both at the population and cell levels. In harmony with these data a decrease in the specific activity of DNA was measured in the PCA-insoluble fraction of tumour cells (Table 2).

Simultaneously with the decreasing rate of DNA labelling the same tendency for RNA and protein synthesis could be observed (Table 2). The question arises as to whether differences in the labelling of the macromolecules reflect differences in the synthesis. However, the data demonstrating a slower decrease in the labelling

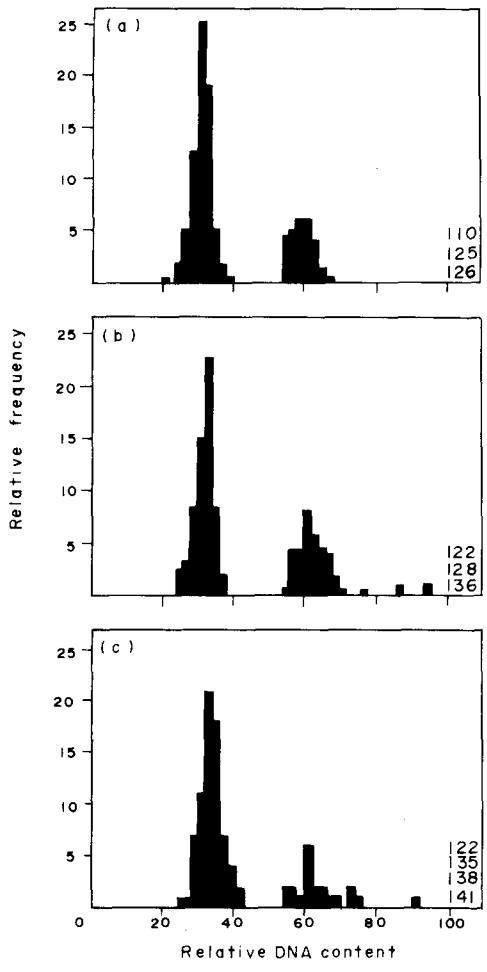

Fig. 5. Cytophotometric study on the relative DNA content of non-labelled NK/Ly ascites tumour cells after single (a—7-day-old, b—14-day-old tumour) and repeated (c—14-day-old tumour) ^{3}H-thymidine injections.

of the acid soluble fraction (nucleotide and aminoacid pool) than in that of the biopolymers seem to suggest that precursors are present in sufficient amount (Table 2). In contrast with the steady DNA content (13–15 pg/cell) the RNA and protein content per cell gradually increased after transplantation.

Measurements on RNA polymerase activity at various days after tumour transplantation give evidence that during enlargement of the tumour mass the RNA synthesis decreases at the transcriptional level (Fig. 6). In the presence of magnesium the incorporation of ^{3}H-UTP was three fold higher in the 3-day-old tumour cell nuclei than in the 8–14-day-old one. Manganese activated RNA polymerase also operated at a lower level in the slowly proliferating tumours.

Since gene functions are controlled by chromatin proteins it was presumed that chromatin proteins show differences in their synthetic rate at various days after tumour transplantation where cells revealed decreasing rate of nucleic acid synthesis. Table 3 shows the specific activity of various protein fractions after 1 hr labelling with ^{3}H-lysine in the 3 and 10-day-old tumours. The lower incorporation into the 10-day-old tumours might be brought about by factors other than synthetic capacity (differences in precursor uptake, etc.). Therefore, specific activity of the nuclear protein fractions were related to the specific activity of proteins in whole cells as has been proposed by Cholon and Studzinski [36]. The figures of the relative specific activity show

Table 2. Changes in the quantity and synthesis of nucleic acids and protein at different growth stages of NK/Ly ascites tumour

Tumour age (day)	2	4	7	10	14
pg RNA/cell	26.68 ±2.18	32.21 ±2.21	33.7 ±9.5	46.84 ±12.0	46.6 ±8.38
pg protein/cell	144.4 ±42.1	167.46 ±20.2	180.3 ±19.35	234.77 ±37.2	227.6 ±63.09
dpm/mg DNA $\times 10^{-3}$	27570 ±9860	11000 ±1210	3530 ±945	1760 ±328	745 ±328
dpm/mg RNA $\times^{-3}$	39201 ±7600	7663 ±1800	2860 ±230	1387 ±258	1026 ±288
dpm/mg protein $\times 10^{-3}$		210450 ±69800	92369 ±16900	58982 ±3275	28406 ±12400
s.a. nucleotide pool*	6.13	7.04	10.48	23.01	118.12
s.a. of DNA / s.a. nucleotide pool†	28.06	60.34	32.09	46.67	
s.a. of RNA / s.a. aminoacid pool‡		0.041	0.056	0.115	0.285
s.a. protein					

*After ^{3}H-thymidine injection.
†After ^{3}H-uridine injection.
‡After ^{3}H-leucine injection.

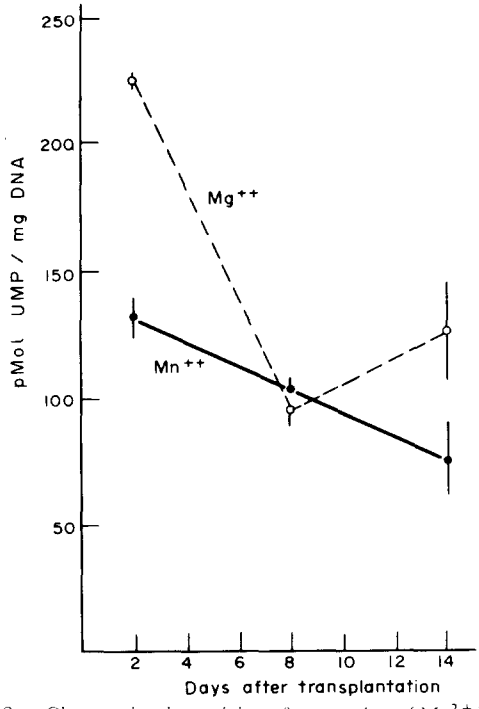

Fig. 6. Changes in the activity of magnesium (Mg^{2+}) and manganese (Mn^{2+}) activated RNA polymerase at different growth stages of NK/Ly ascites tumour.

that labelling of nuclear proteins is definitely lower than whole cell proteins, however, after 24 hr they tend to increase—except chromatin proteins extracted with 0.35 M NaCl from 3-day-old tumour—indicating a slower turnover for chromatin proteins as for other cellular proteins.

Histones and residual acidic proteins were separated on SDS-polyacrylamide gel electrophoresis. The residual acidic proteins isolated from the 3-day-old tumours showed a high level of labelling in protein fractions with a mol. wt of about 40–50,000. On the contrary, in the 10-day-old tumours, the predominant incorporation of ^{3}H-lysine was observed in a much lower mol. wt region (Fig. 7a and b). Studies on histones isolated from 3-day-old tumour demonstrated that labelling in F_3 and F_{2b} surpassed all other fractions, however, in the case of the 10-day-old tumour F_{2a_1} and F_{2a_2} fractions were equally labelled as well (Fig. 8a and b).

Since binding of ethidiumbromide to DNA is prevented by chromatin proteins—mainly by F_1 histone—the interaction between DNA and protein could be estimated by measuring fluorescence intensity of ethidiumbromide which had been complexed to chromatin [34]. The lower ethidiumbromide fluorescency observed in the chromatin of 10-day-old tumour compared to the one isolated from the 3-day-old tumour (Table 4) may be regarded as an indication that in the slowly proliferating cells the chromatin proteins are more extensively bound to DNA i.e. the size of the available free segments in the DNA has been decreased at advanced growth stages of the tumour.

Electronmicroscopic study

The NK/Ly ascitic tumour cells are usually spherical with few microvilli on their surface. The karyoplasm of oval or bean-shaped nuclei is medium electron dense. Using OsO_4 fixation some tumour cells of 2–4-day-old tumours showed an even distribution of chromatin without condensation either around the nucleolus or along the nuclear membrane (Fig. 9). In addition to the great number of free ribosomes only few rough surfaced endoplasmic reticulum (rEr) could be observed. The Golgi area was usually broader and the cytoplasm contained some lysosomes and lipid droplets in the advanced age of the tumour. The double fixation maintained the structure better than the OsO_4 and the marginised chromatin was unanimously present in all cells. The electronmicroscopic cytochemical studies revealed further differences in the ultrastructural organization of cell nucleus and cell surface. The EDTA "staining" of 10–14-

Table 3. Incorporation of ^{3}H-lysine into nuclear protein fractions isolated from 3 or 10 days old tumour

	Specific activity counts/min $\times 10^3$ per mg protein		Relative specific activity*					
Age of tumour	3 days	10 days	3 days			10 days		
Labelling time	1 hr	1 hr	1 hr	6 hr	24 hr	1 hr	6 hr	24 hr
Whole cells	72.5 ± 1.2	34.1 ± 1.5	1.0	1.0	1.0	1.0	1.0	1.0
Nuclear fractions								
−0.15 M NaCl soluble	39.5 ± 0.7	21.2 ± 0.9	0.54	0.62	0.73	0.62	0.82	0.97
−0.35 M NaCl soluble	53.3 ± 0.8	27.2 ± 1.4	0.73	0.74	0.53	0.79	1.01	0.89
Histones	62.6 ± 1.6	28.7 ± 1.0	0.86	0.75	0.97	0.84	0.92	1.09
Residual acidic proteins	44.5 ± 1.2	24.8 ± 0.8	0.61	0.69	0.89	0.72	0.72	0.74

*Relative specific activity $= \frac{\text{spec. act. of protein fraction}}{\text{spec. act. of whole cell protein}}$.

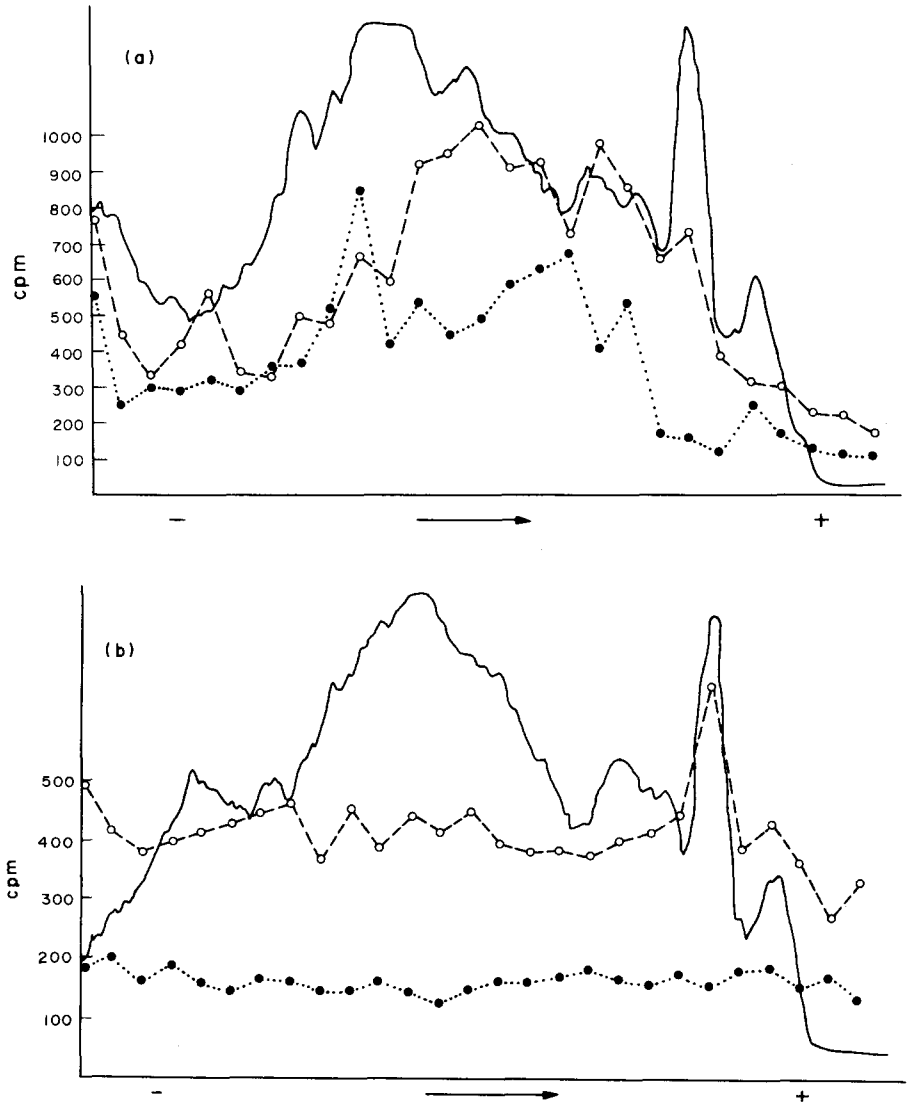

Fig. 7. Gel electrophoretic pattern and radioactivity profiles of nuclear residual proteins.

——— *Densitometric tracings at* 575 nm.
-○-○-○- *Radioactivity after labelling with* ^{3}H-*lysine for* 1 hr.
-●-●-●- *Radioactivity after labelling with* ^{3}H-*lysine for* 24 hr.
a—3-*day-old tumour*, 150 μg *protein.*
b—10-*day-old tumour*, 350 μg *protein.*

day-old tumour was quite regular showing the RNP containing structures (e.g. nucleolus) with high density, and the DNP structures (chromatin) appeared with medium density—as grey fields (Fig. 10). On the contrary, in the 2–4-day-old cells the RNP and DNP structures were equally stained as electron dense areas. The presence of a regularly stained young leukocyte rules out the possibility of a methodical error (Fig. 11).

The cell surface was studied by ruthenium red staining. The cell coat of 2-day-old tumour cells proved to be continuous while with the tumour age it became more and more discontinuous. The thickness of the cell coat at 2-day-old tumour was 470 Å ± 188 Å (extreme values: 163 Å–920 Å) and at 10-day-old tumour it was 160 Å ± 46Å (extreme values: 105 Å–290 Å) (Figs. 12 and 13).

DISCUSSION

The cell kinetic data revealed a tendency of the growth rate of NK/Ly ascitic tumour to slow down from the time of transplantation, as has been found in other ascitic tumours [1–13, 37]. The growth rate was modified by the number but not by the age of the inoculated cells. This may suggest that despite the decreasing rate of cell proliferation during the tumour growth, the

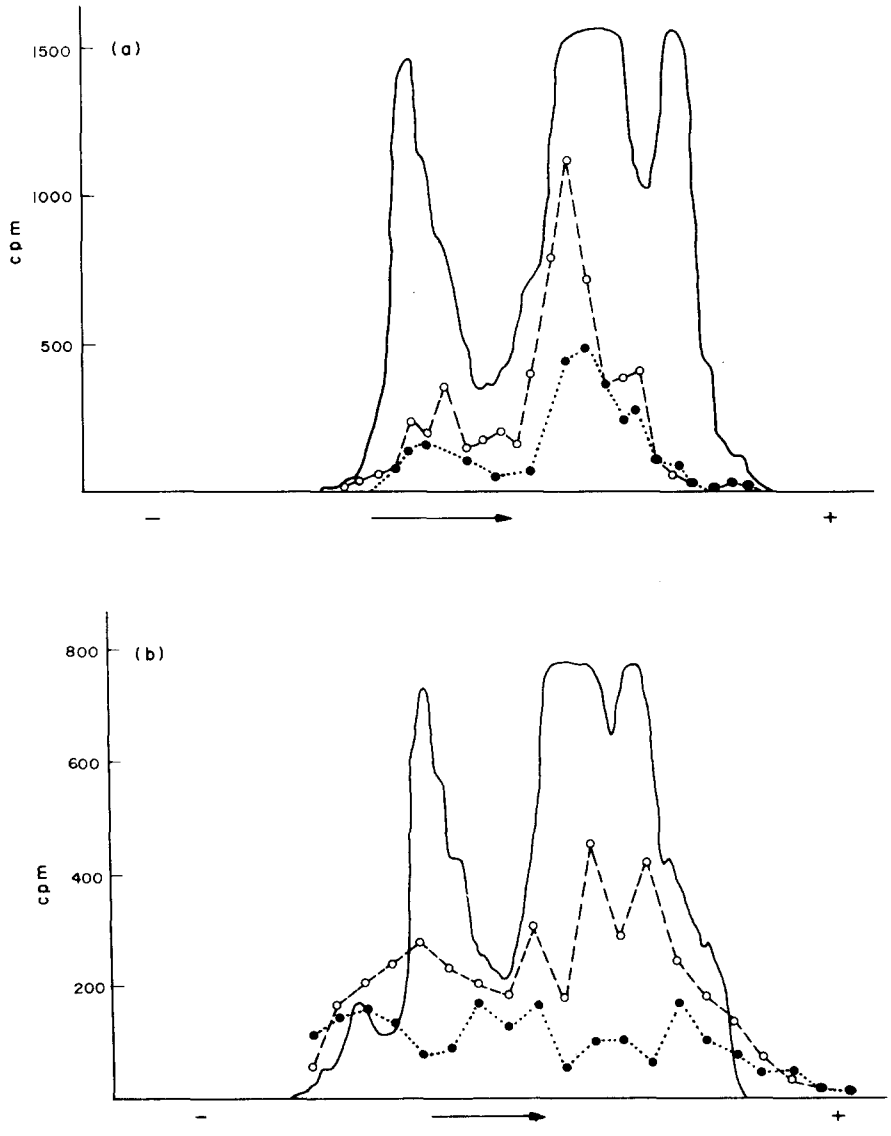

Fig. 8. Gel electrophoretic pattern and radioactivity profiles of histones.
——— *Densitometric tracings at* 575 nm.
● ● ● *Radioactivity after labelling with* ^{3}*H-lysine for* 1 hr.
○ ○ ○ *Radioactivity after labelling with* ^{3}*H-lysine for* 24 hr.
a—3-*day-old tumour*, 150 μg *protein.*
b—10-*day-old tumour*, 350 μg *protein.*

cells regained their growth capacity in a new environment. The main reason for growth retardation was a remarkable increase both in the length and the distribution of cell cycle time and its phases. The rate of cell loss increased slightly between 7 and 10 days.

The growth fraction decreased with tumour age indicating that some cells are blocked in the interphase. The relative amount of DNA at 14 days indicated that most of these cells had the G_1 DNA content, but 19% of them had a DNA content of the G_2 state. It may be concluded that cells were able to interrupt their cycle both in G_1 and G_2. The G_1 arrest of normal and tumour

Table 4. Fluorescency of ethidiumbromide in the presence of desoxyribonucleoprotein isolated from 3 and 10 days old NK/Ly ascites tumour

Tumour age	$I-I_0$*
3 days	49.0†
	53.0
10 days	28.5
	21.0

*I_0 = fluorescency of ethidiumbromide alone.
I = fluorescency of DNP-ethidiumbromide complex.
†Fluorescent intensity in arbitrary unit related to 1 OD_{260}.

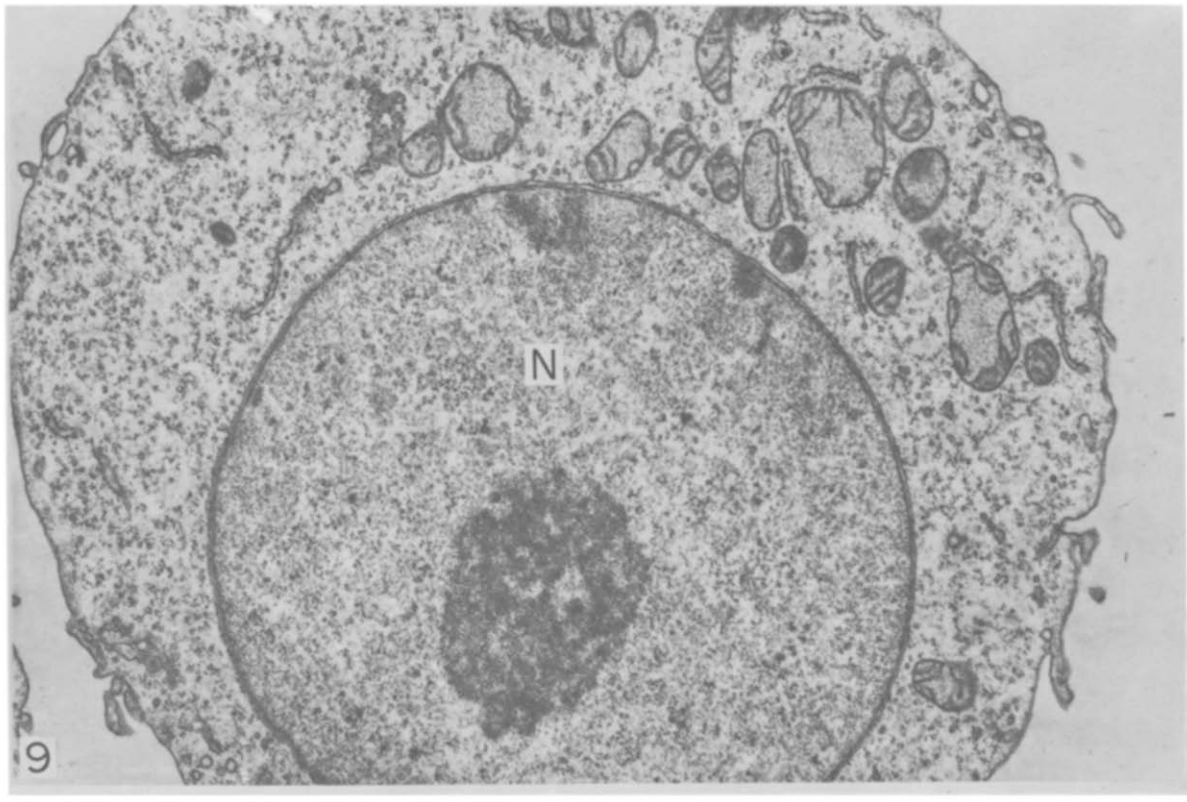

Fig. 9. Four-day-old NK/Ly ascites tumour cell. Note the homogeneous distribution of nuclear material (N) after OsO_4 fixation. × 14,650.

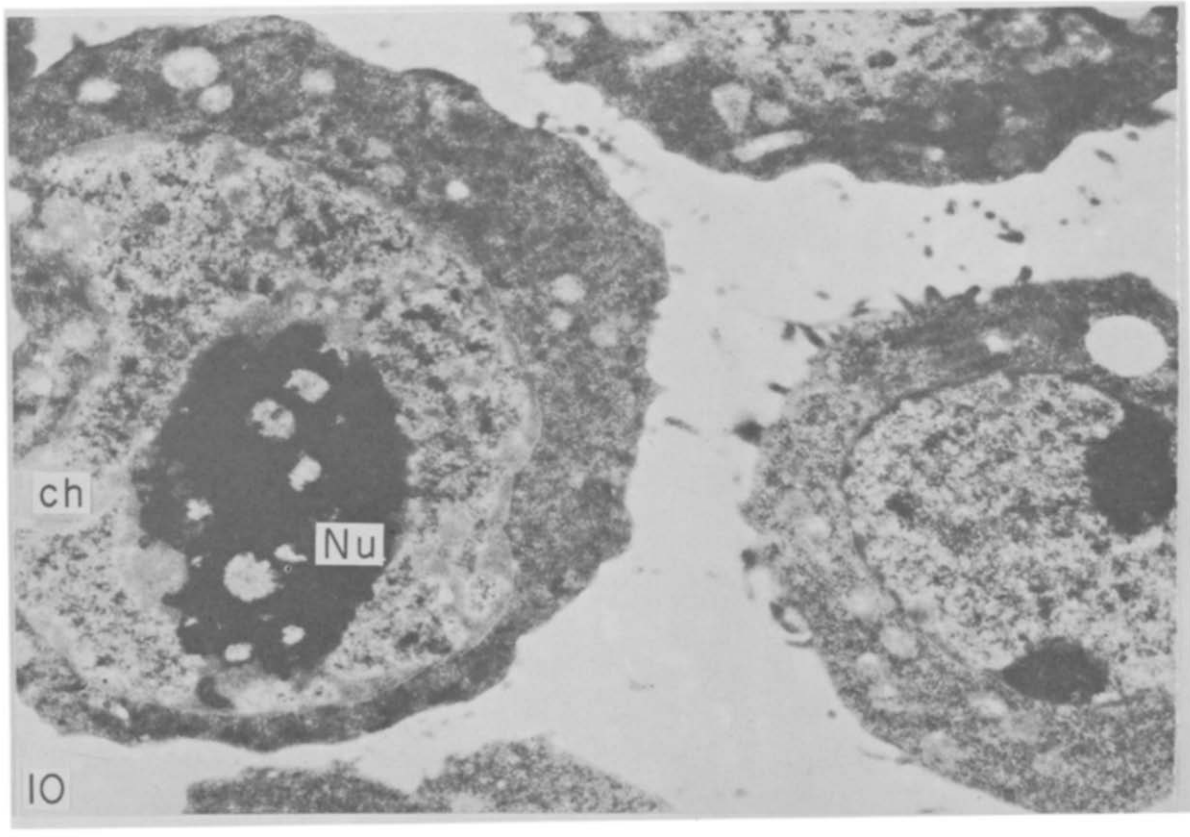

Fig. 10. Fourteen-day-old NK/Ly ascites tumour cells after EDTA "staining". The nucleoli (Nu) appear as electron dense fields, while the chromatin (ch) as more electronlucent grey fields. × 18,000.

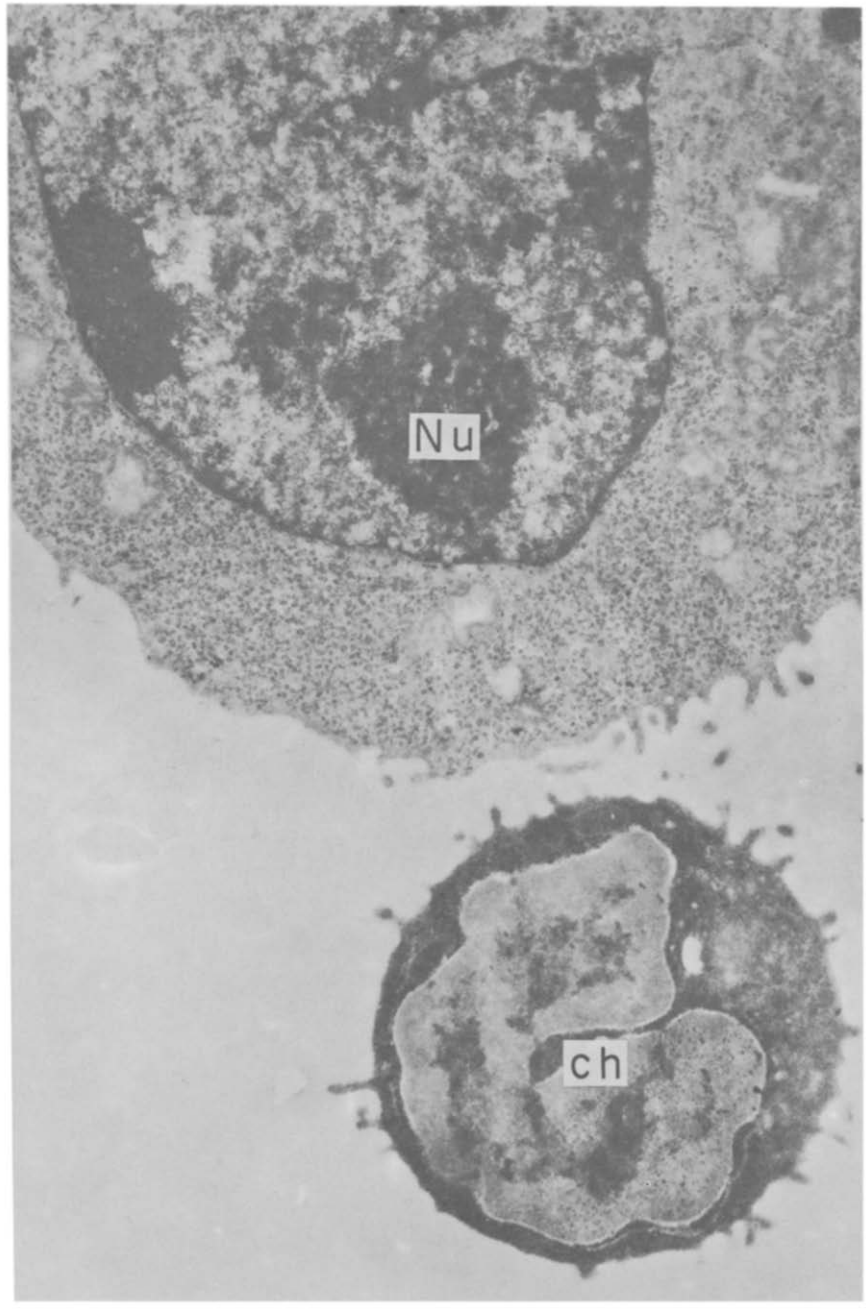

Fig. 11. *Two-day-old NK/Ly ascites tumour. The nucleolus (Nu) and chromatin (ch) of the tumour cell (above) have similar electron dense appearance, while the young leucocyte (below) shows the expected staining following EDTA treatment.* ×18,000.

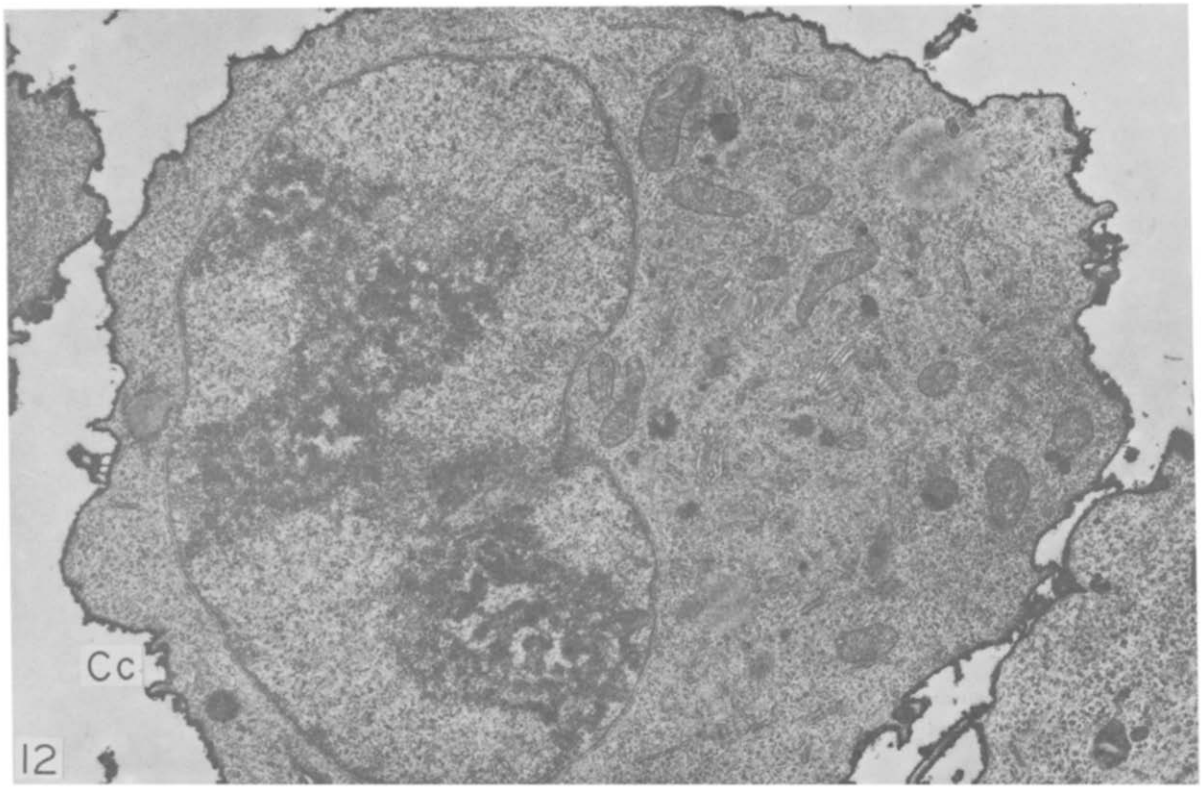

Fig. 12. *Two-day-old NK/Ly ascites tumour cell. The cell coat (Cc) is stained by ruthenium red.* × 12,500.

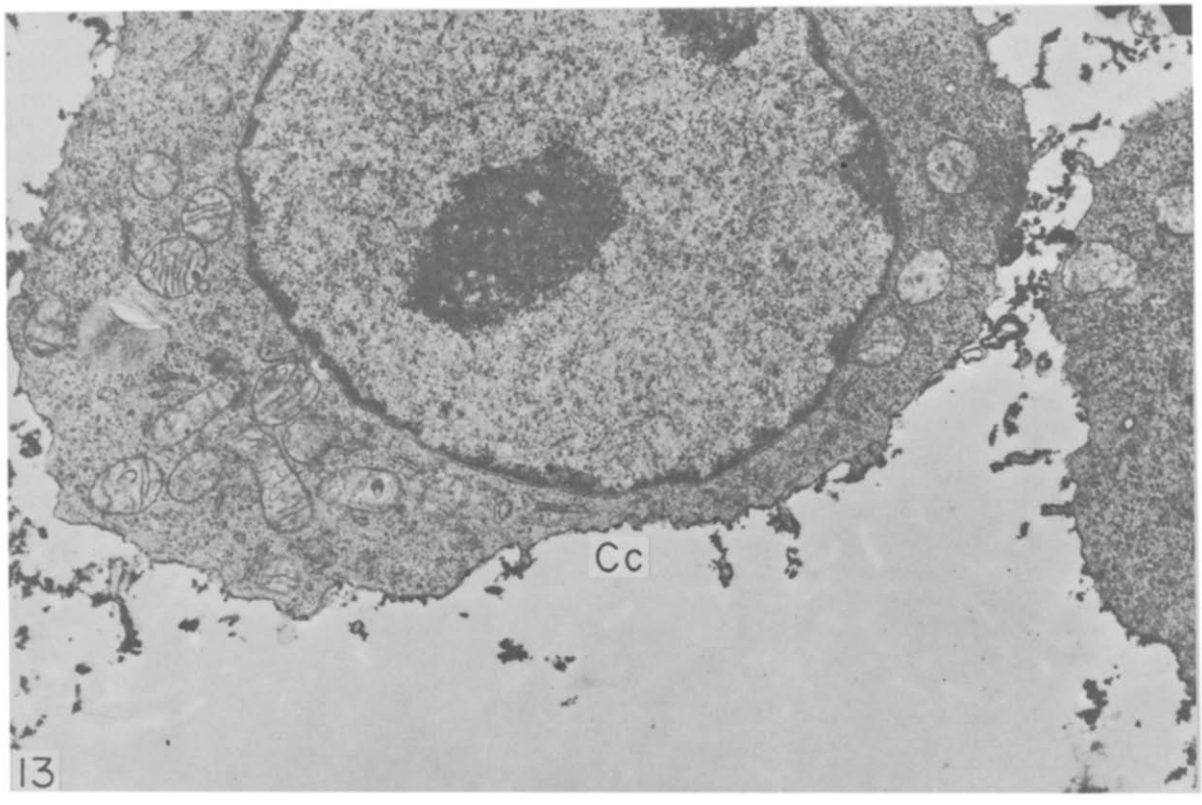

Fig. 13. *Fourteen-day-old NK/ly ascites tumour cell. The cell coat (Cc) stained by ruthenium red became discontinuous and thin compared to the 2-day-old tumour cell.* × 12,500.

cells is well known [38] and date confirming the possibility of G_2 arrest in ascites tumours is also available [39]. However, owing to the presence of tetraploid cells in the NK/Ly ascitic tumour, it is uncertain whether the unlabelled cells having a G_2 DNA content after repeated ^{3}H-thymidine injections are tetraploid cells in G_1 phase or diploid cells in G_2.

The RNA and protein content per cell showed steady increase after the transplantation as a result either of the enhanced rate of RNA and protein synthesis or the declining rate of their catabolic processes compared to synthetic ones. The latter explanation seems to be more plausible since a gradually decreasing rate of both RNA and protein synthesis during tumour growth could be demonstrated. Similar changes in RNA and protein synthesis have been observed by Harris *et al.* [40], using Ehrlich ascites tumour. The decrease in ^{3}H-thymidine labelled cells studied by autoradiography at population level served further evidence to the alteration of DNA metabolism. Decreasing activity of DNA polymerase has also been detected autoradiographically [41].

These events accompanying tumour growth may be regulated at the chromatin level. Studies on chromatin proteins demonstrated differences in the nuclear metabolism between "fast" and "slowly" proliferating cell populations of NK/Ly ascites tumour.

These differences appeared in the various labelling patterns of the chromatin proteins separated on polyacrylamid gel electrophoresis. In agreement with the report of Wisenthal and Ruddon [42] the "fast" proliferating cells could be characterized by preferential labelling in the high mol. wt region of the nuclear residual acidic proteins. However, at later stages when cell proliferation decreased significantly in the same tumour, the preferential labelling took place in a residual acidic protein fraction with a much lower molecular weight. These data seem to indicate the implication of high molecular weight proteins in maintaining the fast proliferating state of the cell population. Since certain histones (F2a2 and F2a1) showed a relatively higher labelling in 10-day-old than in 3-day-old tumours it may be presumed that these histones play a critical role in the slow proliferating property of these ascitic tumour cells. As a further conclusion to the present studies based on the decreased RNA polymerase activity and the lower ethidiumbromide binding to DNA in the 10-day-old tumour than in the 3-day-old one may suggest an increasingly repressed state of chromatin during tumour growth.

According to the electronmicroscopic studies on the ascitic tumour cells at different growth stage changes in the density of cytoplasm, in the appearance of mitochondria, rough surfaced endoplasmic reticulum and lipid droplets were observed [43–46]. In the case of NK/Ly ascites tumour one of the most striking findings was the lack of preferential RNP staining, i.e. the staining of chromatin besides the RNP containing structures using Bernhard's method [25] in the cells of 2–4 days old ascites tumour. A likely explanation for this phenomenon would be a change in the structure of chromatin or in the relationship between the nucleolus and associated chromatin with tumour age. Another important alteration was observed on the cell surface. The trypsin-sensitive cell coat stained by ruthenium red seemed to be more discontinuous and thin with the age of the tumour.

In this study the NK/Ly ascites tumour served as a tool to obtain more data on tumour growth. It is widely known that the deceleration of growth rate occurs in growing cell populations both *in vitro* and *in vivo*. Different changes involved in this process could be detected but an order of events may be suggested only hypothetically. It seems to us that the morphological and biochemical observed alterations of chromatin may be responsible for the decreased synthetic rate of macromolecules and for the cell kinetic changes. However, it may be presumed that before the modification of chromatin, other cell components may be altered e.g. the cell surface, which results in the suppression of the genetic material [47].

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Studies of the Growth of an Ascitic Tumour. II. A System to Study the Tumour-Age Dependent Effect of Antitumour Agents

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Abstract—*Tumour-age dependent activity of an antimetabolite and some alkylating agents was studied using a newly described* in vivo *system. Treatment was performed at different growth stages (i.e. on the* 4th *and* 10th *day after transplantation) on NK/Ly ascites tumour, characterized by different rates of proliferation. The tumour growth inhibitory effect of the drugs was measured on the* 7th *day after the retransplantation of the treated cells. According to the data, the cytostatic agents could be classified into three categories: (a) those having the same effect on the two populations: cyclophosphamide, Lycurim, DMDAI, CCNU; (b) those having greater effect on the fast proliferating* 4*-day-old tumour:* 5*-FU, DBD. DAD, MeCCNU; (c) those having greater effect on the slowly proliferating* 10 *day-old tumour: BCNU.*

INTRODUCTION

THE STUDY of the correlation between the tumour growth rate and therapeutic sensitivity is one of the broadly investigated and discussed approaches in cancer chemotherapy. Series of experiments have been carried out in this field applying various *in vivo* and *in vitro* test-systems [1–12].

Our previous investigations on the growth of an ascites tumour allowed us to design a new model for studying the tumour-age dependent activity of various antitumour agents under *in vivo* circumstances. It has been described that the growth rate of NK/Ly ascites tumour decreased with the age of the tumour which was accompanied by cell kinetic, morphological and biochemical alterations such as the increase of duration and distribution of cell cycle time, the decrease of the ratio of proliferating cells, elevated RNA and protein content of tumour cells, decreased synthetic rate of biopolymers and changes in the structure and function of chromatin [13]. All of these data suggest a progressively but reversibly repressed genetic activity during the tumour growth. According to the results the 4 and 10 days old tumours were regarded as "fast" and "slowly" proliferating populations respectively (Table 1). The purpose of recent investigations was to compare the drug sensitivity of the 4 and 10 days old tumours using mainly alkylating agents.

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Table 1. *Cell kinetic and biochemical parameters of NK/Ly ascites tumour at* 4 *and* 10 *days after transplantation*

	4-day-old	10-day-old
Doubling time (hr)	33.12	132.58
Median cell cycle time (hr)	30.6	56.7
Growth fraction	0.88	0.68
Thymidin index (%)	55.0	36.5
Cell loss factor	0.02	0.45
dpm/mg DNA $\times 10^{-3}$	11000	1760
dpm/mg RNA $\times 10^{-3}$	7663	1387
dpm/mg protein $\times 10^{-3}$	210450	58982

MATERIAL AND METHODS

The *NK/Ly ascites tumour* was used throughout this study. The serial transplantation was performed by the injection of 5×10^6 tumour cells into the peritoneal cavity of noninbred CFLP mice (Laboratory Animals Breeding Center, Gödöllö, Hungary).

The tumour-bearing mice were treated by various drugs intraperitoneally on the 4th and 10th days following transplantation. One hour later their abdomen was opened, the ascites tumour was removed, the cells were counted by haematocytometer and the cell concentration was adjusted to 2×10^7/ml, 10^7 cells (0.5 ml) were retransplanted into healthy mice i.p. and the total number of cells was registered at different intervals thereafter. Four to six mice were used on days 2 and 4, while 6–8 mice were killed on day 7 after transplantation.

The *growth inhibitory effect* was estimated from the value of the total number of cells obtained on the 7th day after retransplantation. To evaluate the differential effect of a drug on "fast" and "slowly" proliferating cells, an index called *tumour-age factor* was introduced and calculated as follows:

$$\text{Tumour-age factor} = \frac{\text{percentage of growth inhibition of 10-day-old tumour}}{\text{percentage of growth inhibition of 4-day-old tumour}}$$

The *following drugs* were used:

Cyclophosphamide (NSC 26271)
2-bis(2-chloroethyl)amino tetrahydro-2H-1,3,2-oxaphosphorine 2 oxide.
VEB, Jenapharm, Ankerwerk, Rudolstadt, DDR.

BCNU (NSC 409962)*
1,3-bis(2-chloroethyl-)-1-nitrosourea.

MeCCNU (NSC 95441)*
1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea.

CCNU (NSC 79037)*
1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
Division of Cancer Treatment, NCI, U.S.A.
*(Supplied by Dr. Goldin).

5-Fluorouracil (NSC 19893)
Roche Products Ltd., Welwyn Garden City, England

Dibromodulcitol, DBD (NSC 104800)†
1,6-dibromo-1,6-dideoxy-D-dulcitol.

Dianhydrodulcitol, DAD (NSC 132313)†
1,2-5,6-dianhydro-dulcitol.
†Chinoin Chemical and Pharmaceutical Works Ltd., Budapest, Hungary.

DMDAI‡
1,6-dimesyl-2,3-4,5-dianhydro-L-iditol.

Zitostop‡
1,2-5,6-tetra-O-methylsulphonyl-D-mannitol.

Lycurim‡
1,4-di-mesyl-oxiethylamino-1,4-dideoxy-m-erythritol.
‡Institute of Drug Research, Budapest, Hungary.

Cyclophosphamide, 5-fluorouracil and DAD were solved in physiological saline; DBD, DMDAI, Zitostop and Lycurim were suspended in 5% Tween-80 and the nitrosoureas in DMSO-5% Tween 80 (1:9 v/v). The controls were injected with the appropriate solvent.

RESULTS

Figures 1 and 2 demonstrate the regrowth curves of treated and retransplanted cells. The effect of 5-fluorouracil—the only non-alkylating agent among the drugs examined—could be regarded as a control in this system. According to

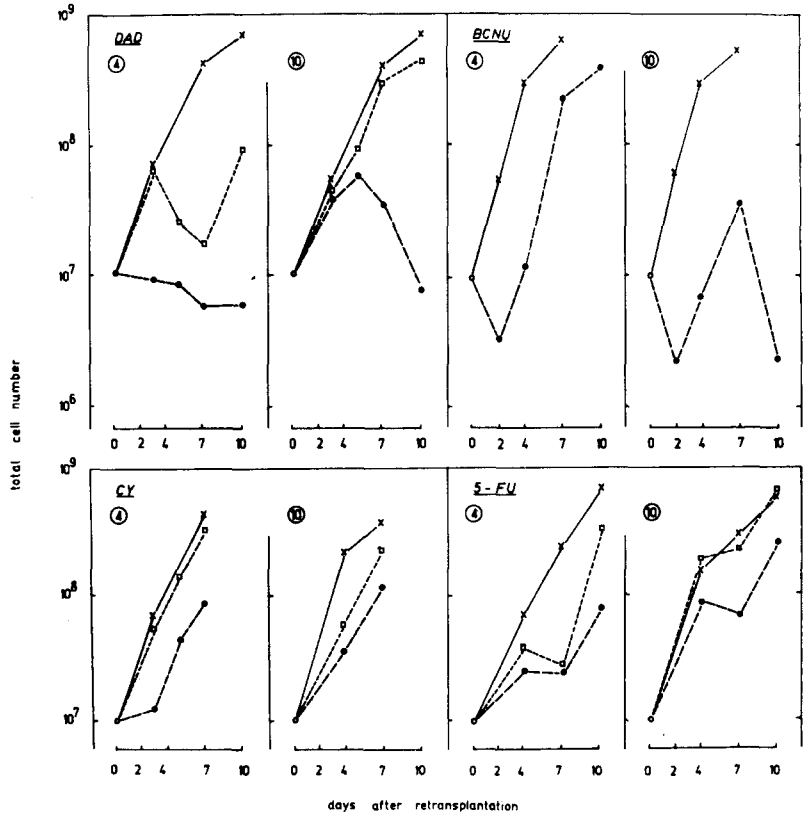

Fig. 1

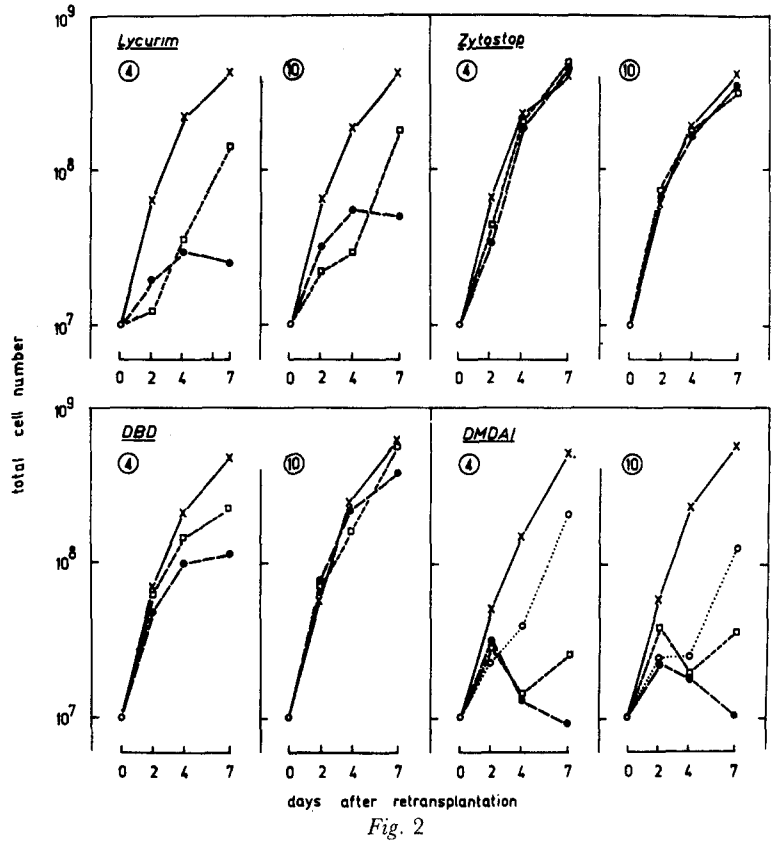

Fig. 2

Figs. 1 *and* 2. *The regrowth curves of treated and untreated NK/Ly ascites tumours. The treatment was performed on the* 4th *and* 10th *days (marks in circles) for* 1 *hr and* 10^7 *cells were retransplanted into healthy mice i.p.*

Control	×-----× ○----○	□·····□	●-----●
DAD		3.5 mg/kg	7.5 mg/kg
BCNU			20 mg/kg
CY		40 mg/kg	80 mg/kg
5-FU		100 mg/kg	500 mg/kg
Lycurim		8 mg/kg	20 mg/kg
Zytostop		500 mg/kg	1250 mg/kg
DBD		140 mg/kg	350 mg/kg
DMDAI	70 mg/kg	140 mg/kg	350 mg/kg

expectation, the "fast" proliferating 4-day-old tumour showed a more pronounced sensitivity against 5-fluorouracil than the 10-day-old one. The shapes of the curves indicate that the cells are still able to complete at least one division after treatment. DBD did not influence the proliferation of the 10-day-old tumour, but the 4-day-old one was affected, showing a dose-dependent inhibition after a temporary control-like growth. The inhibiting effect of DAD, an epoxid derivative of DBD, appeared both on the 4 and 10 days old tumours, but it appeared earlier and lasted longer on the former one. There were no obvious differences in the shape of regrowth curves after cyclophosphamide, DMDAI and Lycurim treatments indicating a similar degree of inhibition of the two cell populations. Higher sensitivity of the 10-day-old tumour was observed only in the case of BCNU.

The result of the BCNU treatment prompted us to get more data not only on the effect of BCNU, but also on the other nitrosoureas, such as CCNU and MeCCNU. Different doses of the drugs were used and the ratio of the total cell number of treated to controls on the 7th day after retransplantation was plotted against the doses. Figure 3 shows the dose–response curves. The MeCCNU was slightly more effective against the 4-day-old tumour as compared to the 10-day-old one. On the contrary the BCNU was much more effective against the slowly proliferating 10-day-old tumour. The CCNU had almost the same effect on the two cell populations. Drawing the curves with regard to tumour age (Fig. 4) it is obvious that real differences could only be seen in the sensitivity of the 4-day-old tumour.

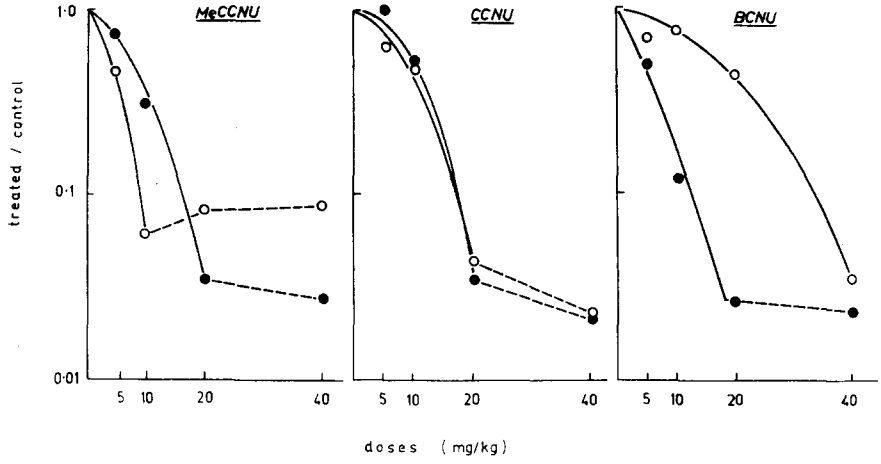

Fig. 3. Dose–response curves of nitrosoureas. The growth inhibiting effect was calculated from the total cell number counted at 7th day after retransplantation. 4-day-old tumour ○----○ 10-day-old tumour ●----● (The values of 4 and 10 days tumours were significantly different— $P<0.05$—at 5–10 mg/kg of MeCCNU and 10–20 mg/kg of BCNU).

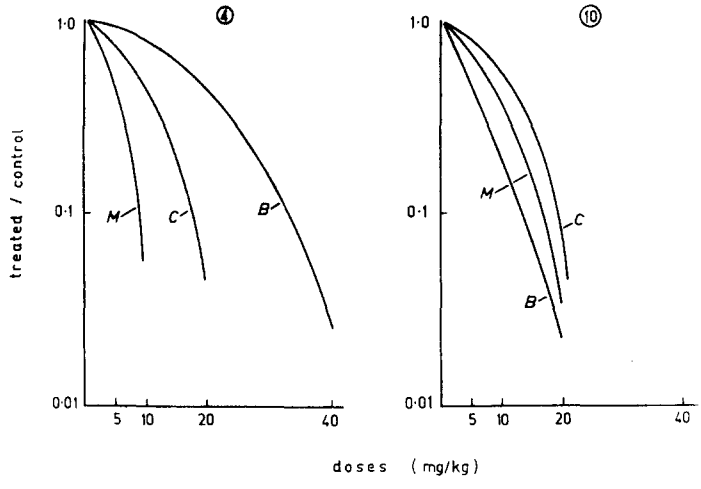

Fig. 4. Dose–response curves of nitrosoureas grouped according to tumour age. (M = MeCCNU, C = CCNU, B = BCNU).

DISCUSSION

There are some observations which stimulate the study of the correlation between tumour growth and therapeutic sensitivity. In this respect one of the important clinical data is the relatively good response of rapidly proliferating human tumours against chemotherapeutic agents [14, 15]. Unfortunately, these highly responsive tumours represent only a small fraction of cancer incidences. It is believed that the presence of a large fraction of proliferating cells, i.e. the high growth fraction, may explain their responsiveness. Another stream of clinical and experimental studies emphasized that the speed of cell proliferation is fairly varied not only in different kinds of tumours, but at different stages of growth of the same tumour [16–19]. It was also proved that the cell kinetic status may play an important role in the therapeutic sensitivity of tumours [1, 20]. Finally, we must mention the kinetic differences between normal and malignant tissues serving a possibility to enhance the selectivity of our treatment [21].

Table 2. Different system for studying the proliferation-dependent effect of chemotherapeutic agents

Author(s)	Proliferating	Non-proliferating	Duration of treatment	Evaluation
Bruce *et al.* [1] Valeriote and Tolen [3]	Transplantable lymphoma	Normal bone marrow	24hr	CFU*
Wilcoff *et al.* [2]	L 1210 leukemia (*in vitro*) exponential phase	L 1210 leukemia (*in vitro*) plateau phase	Various times	*In vivo* bioassay (survival)
Van Putten *et al.* [4]	Normal bone marrow stimulated with radiation	Normal bone marrow	16hr	CFU*
Lin [5]	B lymphocyte stimulated with SRBC	B lymphocyte	4 days	PFU*
Hagemann *et al.* [6]	P 815 × 2 mastocytoma (*in vitro*) exponential phase (*in vivo*) ascites form 2.—5.—10.day solid form 6.—10.—18.day	P 815 × 2 mastocytoma (*in vitro*) plateau phase	15 min	*In vitro* colony
Barranco *et al.* [7] Hahn *et al.* [8]	Chinese hamster ovarian cells (CHO HA 1) (*in vitro*) exponential phase	CHO HA 1 (*in vitro*) plateau phase	1hr	*In vitro* colony
Tobey *et al.* [9]	Chinese hamster ovarian cells in complett F 10 medium	CHO cells in isoleucine-deficient medium	2hr	*In vitro* colony

*CFU = colony forming unit; PFU = plaque forming unit.

As it has been pointed out, various systems were applied to study the correlation between the growth rate and therapeutic sensitivity of tumours. A few of them were compiled on a table to demonstrate not only the variety of the systems but also the different treatment times and the types of evaluation (Table 2). Our system differs from most of these because we compared the sensitivity of the same tumour at different stages of growth. Previously we described some cell kinetic, morphological and biochemical properties of the two (4 and 10 days old) tumour cell populations [13]. It should be emphasized that besides the marked differences most of the cells were in proliferating state in both populations.

Our tumour-age dependent system was based on the observation that the ascites tumour cells derived from tumours of different age retained their proliferative capacity and putting them into a new environment (retransplantation), they are able to grow with the same speed. It means that 10^7 retransplanted cells from 4 and 10 days old tumours produce the same growth curve. The effect of drugs on the two cell populations with different size seems to be easy to compare, since it had been demonstrated that the cell killing effect of the cytostatic drugs follows the primary kinetic order [15]. The tumour inhibiting effect of the cytostatic agents may vary depending on the duration of treatment as it has been shown by Hahn *et al.* [22]. In our case, the one hour treatment time was applied partly to avoid the repair of the drug-caused damage before the retransplantation and partly it was based on our previous data suggesting maximal or near maximal uptake of DBD and DAD after the 1hr treatment [23].

Table 3 summarizes the growth inhibitory effect of the different antitumour agents and the calculated tumour-age factor. These data allowed us to classify the effect of drugs studied into three categories:

1. Those which had no substantially different effect on the fast and slowly proliferating populations—cyclophosphamide, Lycurim, DMDAI, CCNU;
2. Those which were more effective against the fast proliferating tumour cell population—5-FU, DBD, DAD, MeCCNU;
3. The only drug inhibiting the growth of the slowly proliferating 10 days old tumour better than that of the 4 days old one was BCNU.

5-FU produced the expected result regarding its inhibitory effect on thymidilate synthetase to be essential for DNA synthesis. The primary targets of DBD and DAD are the chromosomal proteins [24, 25]. It is interesting to recall our observation that the metabolic state of chromosomal proteins is changing during the tumour growth [13]. These data also suggest that there is a correlation between the proliferation de-

Table 3. The effect of various chemotherapeutic agents at different age of NK/Ly ascites tumour

Drug	Dose (mg/kg)	Growth inhibition* 4 day	Growth inhibition* 10 day	Tumour-age factor†
5-FU	100	82.3 (20.2)‡	11.2 (12.4)	0.14
	500	89.0 (19.6)	68.5 (15.2)	0.77
DBD	140	45.9 (20.0)	5.7 (7.4)	0.12
	350	72.3 (18.2)	36.2 (16.8)	0.50
DAD	1.5	39.8 (16.8)	8.8 (14.2)	0.22
	3.7	92.5 (19.6)	33.2 (18.5)	0.36
	7.5	98.5 (22.7)	95.6 (21.4)	0.97
MeCCNU	5	63.6 (20.8)	40.3 (20.9)	0.63
	10	94.1 (21.6)	72.0 (18.3)	0.77
	20	92.3 (22.5)	96.7 (21.0)	1.05
Lycurim	8	66.6 (17.0)	58.4 (15.4)	0.88
	20	94.3 (19.0)	88.3 (20.3)	0.94
Cyclophosphamide	40	22.5 (13.4)	32.0 (12.4)	
	80	80.5 (22.3)	79.0 (19.0)	0.98
DMDAI	70	59.7 (12.5)	77.6 (14.6)	1.30
	140	94.9 (19.9)	93.6 (16.9)	0.99
	350	98.2 (22.8)	98.2 (16.2)	1.00
CCNU	10	51.7 (16.4)	46.7 (23.2)	0.90
	20	95.6 (24.4)	96.4 (18.7)	1.01
BCNU	10	28.2 (25.0)	89.6 (19.2)	3.17
	20	58.2 (24.0)	97.3 (22.5)	1.67
	40	95.9 (25.0)	98.7 (19.0)	1.03

*Growth inhibition $= 1 - \frac{\text{total cell number of treated}}{\text{total cell number of control}} \times 100.$

†Tumour-age factor $= \frac{\text{growth inhibition (10 day)}}{\text{growth inhibition (4 day)}}.$

‡Variation in the growth inhibition—expressed as the percentage of the growth inhibition—has been estimated according to the equation:

$$\text{Variation} = \pm \sqrt{\frac{A_1^2}{n_1^2} + \frac{A_2^2}{n_1^2}}.$$

where A_1 and A_2 are the ratio of standard deviation to mean value of total cell number in control and treated groups; n_1 and n_2 are the number of mice in respective groups.

pendent activity of DBD and DAD and their intracellular effect.

Cyclophosphamide is known to be more effective against proliferative cells [1, 4]. This information has derived from studies using two "sharply" different populations: proliferating and non-proliferating ones. But in our system both populations were mainly composed of proliferating cells and there was no substantial difference in their sensitivity against Cyclophosphamide. Lycurim was proved to be an effective antineoplastic and immunosuppressive agent in clinical practice as well [26–28]. DMDAI, having both mezil and epoxy groups in its chemical structure, showed a strong antitumour effect experimentally [29, 30]. Neither Lycurim nor DMDAI showed proliferation-dependent effect in our system.

The nitrosoureas behaved quite differently in our tumour-age dependent system—basically against the 4-day-old tumour—which could be the result of the different repair capacity of the damaged cells. From the therapeutic point of view the "preferential" effect of BCNU on a slowly proliferating population seems to be very important because of the shortage of such drugs in our therapeutic regimen. Theoretically, it may mean that with the help of BCNU we could influence the tumours with low growth fractions and perhaps increase the more selective cell-killing between the tumour and the critical (usually rapidly proliferating) normal tissues. Concerning the proliferation dependent effect of BCNU our data is supporting the results of others [6–9], but there are also contradictory data [4, 31]. The advantage of the dose–response curve is clearly seen from data of Table 3, since the value of tumour-age factor approached 1.0 with elevating doses.

From these data it could be concluded that the

tumour inhibitory effect of certain alkylating agents is different, while that of others is similar at various stages of tumour growth. It is felt that if the growth stage of a human tumour to be treated could be judged, the use of the tumour-age factor may be a help in selecting the most promising drug or drug-combination. There is no need to emphasize that the cell kinetic status is an important—but only one of the important—aspects of cancer treatment. We are still far from the causative therapy of cancer, but our increasing knowledge on the correlation between drug effect and tumour growth rate, the cell kinetic complexity of a cell population, the mechanisms operating behind the change of growth rate, and the mechanisms regulating the tumour growth are a hopeful way of keeping cancer under our control.

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Oestrogen Receptors in Transplantable Ovary-Independent, Mammary Tumours of the Rat

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Abstract— *Oestrogen receptor activity (available cytoplasmic binding sites) has been determined in three lines of transplantable rat mammary tumours. At sacrifice, the plasma concentrations of oestradiol-17B and prolactin were determined by radioimmunoassay.*

Low levels of receptor activity were detected in two tumour lines but none was detected in the third line.

Receptor levels in these transplantable tumours, which exhibit ovary-independent growth, were compared with those found in ovary-dependent tumours induced by DMBA in random-bred rats. The comparison was performed in (a) intact animals (b) ovariectomised animals and (c) ovariectomised animals subsequently treated with replacement oestrogen. Irrespective of endocrine status, the mean tumour receptor level was significantly higher in the DMBA-induced tumours. However, considerable overlap between the groups occurred, suggesting that there is no clear division in receptor levels between ovary-dependent and ovary-independent tumours.

INTRODUCTION

PREVIOUS studies have shown that amongst rat mammary tumours induced by DMBA (7,12 dimethyl benz(a)anthracene), ovary-dependent tumours contain higher levels of oestrogen-binding activity than do ovary-independent tumours [1–5]. It is our experience that very few DMBA-induced tumours can be shown to be truly ovary-independent. We have, however, been able to study three lines of transplantable mammary tumours which are clearly ovary-independent.

Since the level of oestrogen receptor activity is markedly affected by the endocrine status of the host [6], we have measured receptor levels in these transplantable tumours and compared the values for two tumour lines with those found for DMBA-induced tumours, in various endocrine states.

MATERIAL AND METHODS

Tumours

Three lines (TG3, TG5, TG6) of transplantable tumours were originally set up by Mr. I. W. J. Wallace, F.R.C.S. and Mr. D. Drewitt, B.Sc. of this department, by subcutaneous implantation of explants from each of three DMBA-induced mammary tumours into hosts of an inbred strain of Sprague–Dawley rat ('ADRA' obtained from the Animal Disease Research Association, Moredun, Edinburgh). Histologically, all three parent tumours were adenocarcinomata, but whilst the TG3 and TG5 lines maintained this morphological characteristic, the TG6 line showed a progressive change with the appearance of some fibrosarcomatous elements over the time of study. At the time of use in the present work, the growth of all three tumour lines was ovary-independent. The growth patterns for typical tumours of each type are shown in Fig. 1. Tumours were selected for study over several generations, the average generation being approximately 20 for the TG3 line, 6 for the TG5 line and 4 for the TG6 line.

DMBA-induced tumours were induced by intragastric instillation of DMBA (30 mg) in a random-bred strain of rats (Oxford Laboratories Ltd.). All tumours were allowed to grow until at least 1.5 × 1.5 cm before use for receptor assay or ovariectomy.

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Endocrine manipulation and assessment of ovary-dependence

In order to assess ovary-dependence/independence, the tumours in one group of animals (group a) were measured at intervals with calipers before and after bilateral ovariectomy under ether anaesthesia. Tumour size was expressed as the product of measurements in two dimensions.

For a second group of animals (group b) tumours were measured before and during sequential manipulation by ovariectomy, no treatment for 10 days and oestradiol-17B administration for 6 days (1–5 μg/day s.c. in oil).

Ovary-independent tumours were defined as those tumours which continued to increase in size for the period of study following ovariectomy (usually by >30% over 9–14 days).

Ovary-dependent tumours were classified either as those tumours showing a continuous decrease in size for the period following ovariectomy (usually ≧50% over 9–14 days)—group (a), or where animals were subsequently further treated by oestrogen administration, as those tumours regressing upon ovariectomy and clearly regrowing upon oestrogen administration—group (b).

Tumours showing extensive necrosis, changes due to a large cystic fluid content, or equivocal growth patterns were regarded as unassessable and were excluded.

Tumour receptor concentration in intact and ovariectomised rats bearing transplantable tumours

In an initial experiment, receptor concentrations were determined in the tumours from 23 intact, cycling animals (7 TG3, 10 TG5 and 6 TG6). A further 17 rats bearing 23 transplantable tumours (11 TG3, 11 TG5 and 1 TG6) were ovariectomised and left for 14 days prior to sacrifice and determination of tumour receptor concentration.

Comparison of receptor concentrations in transplantable tumours and DMBA-induced tumours

In a second study, receptor concentrations in 2 lines of transplantable tumours (TG3 and TG5) were compared with those in DMBA-induced tumours in each of 3 endocrine states.

For receptor assay, tumours were excised from:

(a) 21 ovariectomised rats (6 regressing, DMBA-induced tumours and 15 growing, transplantable tumours, 8 TG3, 7 TG5);

(b) 21 rats treated by ovariectomy and subsequent administration of oestradiol-17B (15 DMBA-induced tumours showing regression followed by regrowth, and 6 transplantable tumours showing continuous growth i.e. 1TG3, 5TG5);

(c) 22 intact, cycling rats (10 DMBA-induced tumours, 12 transplantable tumours i.e. 10 TG3, 2 TG5).

Thus in groups (a) and (b), only animals showing clearly ovary-independent growth for a DMBA-induced tumour, or clearly ovary-independent growth for a transplantable tumour were examined. In group (c) the tumour specimen for assay was removed by biopsy (in oestrus, metoestrus or dioestrus, but not proestrus) under ether anaesthesia and the animals were then left approximately 14 days and ovariectomised. However, it was not possible to determine the ovary-dependence/independence of these tumours since the biopsy procedure alone frequently disturbed the growth of the tumour.

Measurement of plasma oestradiol-17B and prolactin concentrations

At sacrifice, the animals were bled through the abdominal aorta under ether anaesthesia. In all rats except group (c), bleeding preceded tumour excision, but in group (c), biopsy specimens of tumour were taken and animals were not sacrificed until approximately 4 weeks later.

Plasma was separated by centrifugation at 4°C and stored at −20°C for radioimmunoassay by methods previously described [7]. The sensitivities of these assays were calculated to be 3 ng prolactin/ml plasma and 0.13 ng oestradiol-17B/100 ml plasma when analyses were conducted on duplicate, 0.1 ml and single, 4.0 ml samples of plasma respectively.

Determination of oestrogen receptor concentration

Tumour tissue, removed either by biopsy under ether anaesthesia [group (c) only] or after killing by exsanguination, was placed in Tris buffer containing dithiothreitol (0.25 M sucrose 10 mM Tris, 1 mM ethylene diamine tetraacetate, 0.5 mM dithiothreitol, pH 8.0) and stored on ice for 1 hr. The concentration of oestrogen receptors in each tumour was measured by a method described previously [6].

Briefly, 600–800 mg tumour was trimmed free of adhering tissue and capsule at 4°C, cut up finely with scissors and homogenized in an ice-cooled tube at the rate of 300 mg/ml buffer, using a Silverson homogenizer (1 × 20 sec, 1 × 15 sec at maximum speed with a 45 sec interval for cooling between bursts). The resulting homogenate was centrifuged at 105,000 × ***g*** for 50 min to yield a clear cytosol. Portions (0.1 ml) of cytosol were incubated, in duplicate, at 4°C with 10 pg [^{3}H]oestradiol-17B (5060 counts/min) and varying quantities of non-radioactive oestradiol-17B (0, 10, 30, 50, 70, 90 and 20,000 pg) in a total volume of 1.2 ml Tris buffer (composition as

Table 1. *Tumour receptor concentrations and plasma hormone levels in intact and ovariectomised rats bearing* 3 *lines of transplanted tumours*

Tumour line	Reproductive state	No. of rats	No. of tumours	Tumour receptor activity			Plasma	
				K_d ($\times 10^{-10}$ M)	P_0 (fmole/mg) (tissue)	P_0, protein (fmole/mg) (protein)	Oestradiol-17β (ng/100 ml)	Prolactin (ng/ml)
	Sensitivity*			—	0.05	1	0.13	3
TG-3	Intact	7	7	0.46 ± 0.12	0.48 ± 0.15	11.5 ± 3.8	0.98 ± 0.52	28 ± 12
	OVX	7	11	0.36 ± 0.06	0.59 ± 0.10	11.3 ± 1.8	0.12 ± 0.02†	4.3 ± 1.4†
TG-5	Intact	10	10	0.54 ± 0.06	0.43 ± 0.11	14.5 ± 6.4	1.61 ± 1.75	32 ± 10
	OVX	9	11	0.43 ± 0.08	0.70 ± 0.16	16.0 ± 3.3	0.16 ($n=2$)	5.4 ± 1.1‡
TG-6	Intact	6	6	0.51 ($n=2$)	0.03 ± 0.02	0.7 ± 0.6	2.09 ± 1.51	39 ± 23
	OVX	1	1	—	0	0	0.01	53

Results are mean values ± 1 standard error.
K_d = dissociation constant of oestradiol-17β binding, P_0 = concentration of oestrogen receptor sites. *Sensitivity defined as described under Materials and Methods.
†$P<0.01$, ‡$P<0.10$ when OVX and intact values compared for same tumour line.

above except that dithiothreitol was omitted). After overnight equilibrium, free oestradiol-17B was removed by the addition of 0.5 ml charcoal suspension (0.15% charcoal, 0.0015% dextran in Tris buffer w/v), further incubation at 4°C for 15 min and centrifugation for 10 min at 4°C.

The protein-bound fraction was decanted into 5 ml organic scintillator solution for determination of ^{3}H activity. After correction for non-specific binding using the mean value for the 20,000 pg tubes, the data was analysed according to Scatchard [8] to derive the dissociation constant of binding (K_d) and the concentration of receptor sites (P_c). The latter was expressed both as fmole binding sites/mg wet tumour and as fmole binding sites/mg cytosol protein, using the protein concentration of the cytosol found on measurement by the method of Lowry, Rosebrough, Farr and Randall [9].

The sensitivity of the method was empirically defined as 0.05 fmole/mg wet tumour or 1 fmole/mg cytosol protein, since a clearly increasing displacement of [^{3}H] oestradiol-17B with increasing mass of non-radioactive oestradiol-17B present was generally observed above this receptor level.

Statistics

The significance of differences in receptor concentrations was evaluated by the Wilcoxon rank test.

RESULTS

Tumour receptor concentrations in intact rats

Low, but detectable levels of oestrogen receptor activity were found in two of the three lines of transplantable tumours, i.e. the TG3 and TG5 lines (Table 1). The dissociation constant of binding was of the order of 0.4×10^{-10} molar in each tumour line and no significant difference in receptor levels was apparent between the two tumour lines. In the third tumour line, (TG6), receptor activity was found in only 2 of 6 tumours examined and the levels detected were extremely low.

Effect of ovariectomy

The efficacy of ovariectomy was confirmed by the reduction of plasma oestradiol-17B concentrations to the level of the sensitivity of the assay (Table 1). Prolactin concentrations were also significantly reduced in 2 of the groups of rats bearing tumours (TG3 and TG5) and the apparent rise in prolactin level in the one rat bearing a TG6 tumour which was examined after ovariectomy is probably a spurious result (Table 1).

Despite these changes in plasma hormone concentrations following ovariectomy, no significant change in receptor level was observed in either TG3 or TG5 lines of tumours (Table 1, Fig. 2).

Comparison of receptor levels in transplantable tumours (ovary-independent) with levels in ovary-dependent, DMBA-induced tumours

Receptor levels in the transplantable tumours were compared with those in DMBA-induced tumours obtained from animals in each of three endocrine states. Firstly, 14 days after ovariectomy, (Fig. 3a), receptor concentrations were significantly ($P<0.05$) higher in DMBA-induced tumours which regressed than in the tumours (non-regressed) of either TG3 or TG5 transplanted line, though there was a considerable overlap between groups. Secondly, when tumour-bearing animals were subjected to ovariectomy and subsequent administration of oestrogen (Fig. 3b), the receptor concentrations in ovary-dependent, DMBA-induced tumours were clearly higher than the concentrations in the ovary-independent, transplantable tumours ($P<0.01$).

Thirdly, receptor concentrations in biopsy specimens from uncharacterised, DMBA-induced tumours were also significantly higher than the values found in transplantable tumours of either TG3 or TG5 line, though again there was a considerable overlap between the two types of tumour. Attempts to separate the biopsied, DMBA-induced tumours into ovary-dependent and independent types by their response to subsequent ovariectomy were unsuccessful: The biopsy procedure alone generally interfered with tumour growth.

DISCUSSION

Oestrogen receptor activity has been detected in low but significant amounts in 2 out of 3 lines of transplantable rat mammary tumours. The two lines containing low receptor levels maintained the morphological appearance of adenocarcinomata whilst the other tumour line without detectable activity showed some tendency to progress towards a sarcoma by the time of the study. Gala and Peck [10] have described two transplantable, mammary tumours, of which one was an adenoma with "high" levels of oestrogen receptor activity, whilst the other was a sarcoma devoid of receptor activity.

At the time of study, all three transplantable tumour lines exhibited ovary-independent growth. Prior to this work, however, at least one of these lines (TG3) had passed through an ovary-

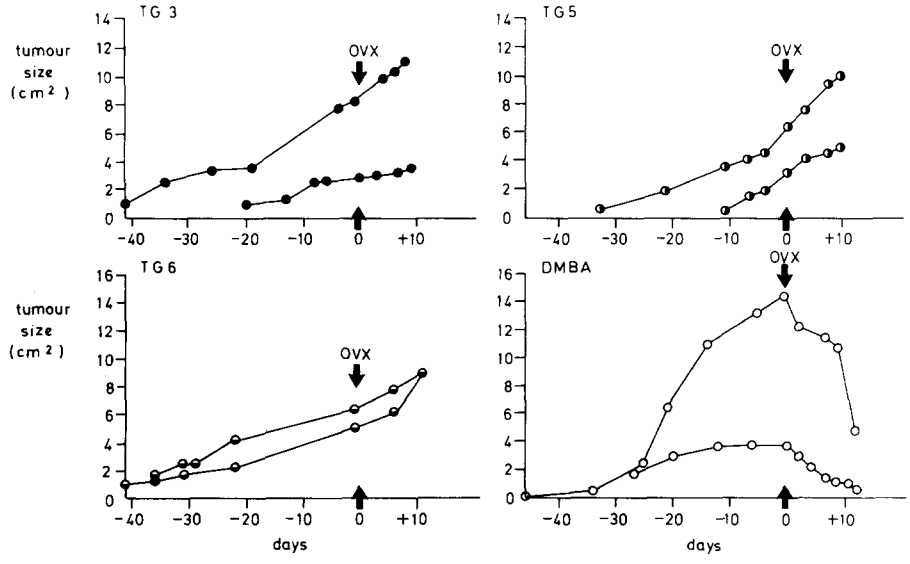

Fig. 1. *The ovary-independent growth of 3 lines of transplantable mammary tumours. Tumour sizes were measured before and after bilateral ovariectomy of 2 rats bearing transplantable tumours, for each tumour line. For comparison, the effect of ovariectomy of the host upon the growth of two typical DMBA-induced tumours is also illustrated. OVX indicates the time of ovariectomy, and measurements are plotted against days pre (−) or post (+) ovariectomy.*

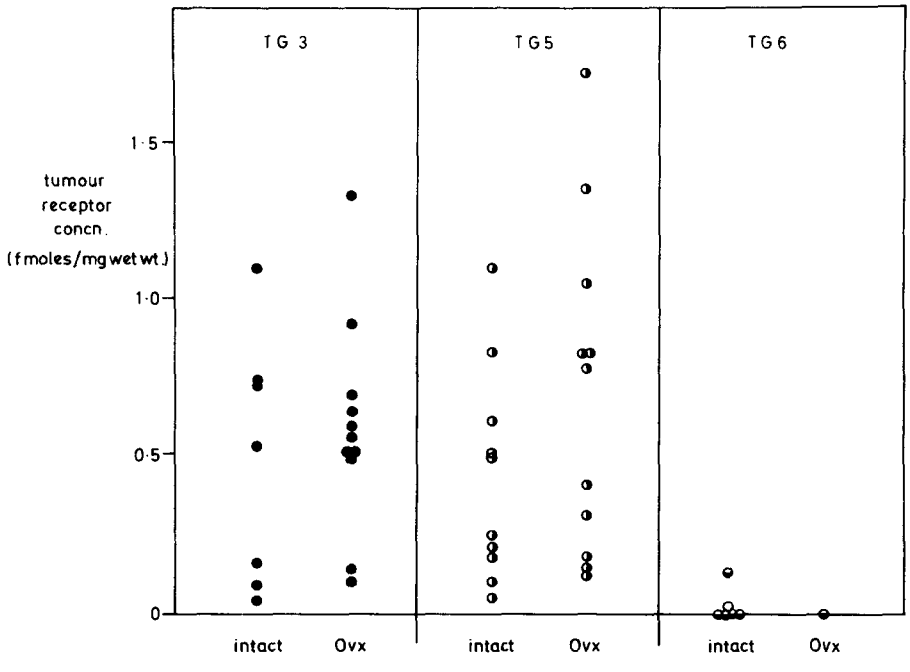

Fig. 2. *Oestrogen receptor concentrations in each of 3 lines of transplantable mammary tumour. For each tumour line, receptor concentrations are shown in the tumours from intact and ovariectomised hosts. Each circle represents the value for a single tumour.*

dependent phase in the very early generations. A similar progression from dependence to independence has been reported for certain strains of transplantable mouse tumour [11] and for other transplantable rat mammary tumours [12, 13].

The mere *presence* of oestrogen receptor activity in a tumour is thus not an indication of ovary-dependence, in agreement with the findings of others [11, 14–16] though as we have previously reported [17], the growth of at least some of these

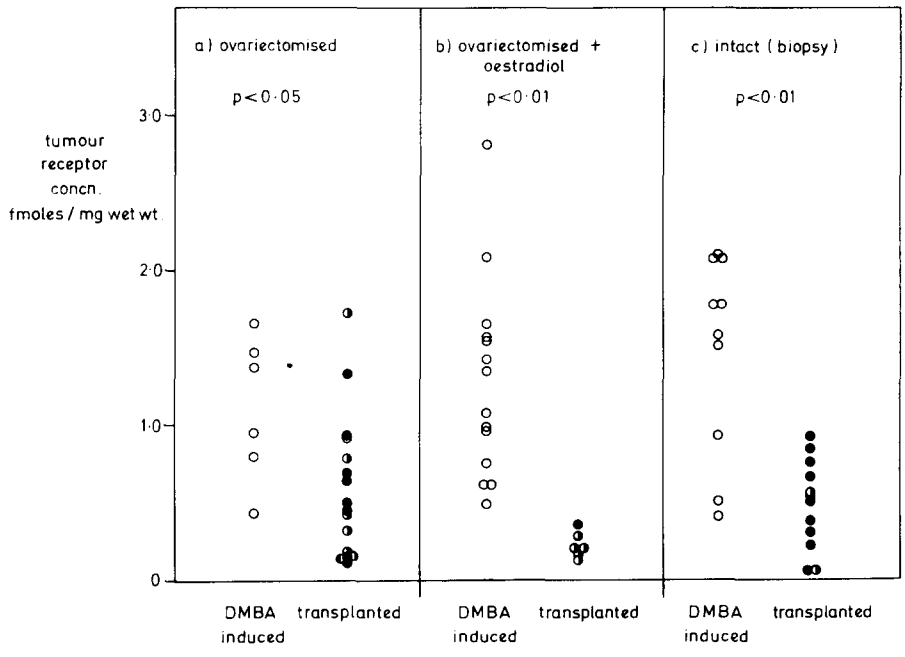

Fig. 3. Oestrogen receptor concentrations in ovary-independent, transplantable tumours were compared with values found in DMBA-induced tumours in each of 3 endocrine states. In (a), ovariectomised rats and (b), rats treated sequentially by ovariectomy and oestradiol administration, open circles denote receptor concentrations from ovary-dependent, *DMBA-induced tumours; in (c), intact animals, open circles represent receptor concentrations in uncharacterised DMBA-induced tumours. Solid circles represent receptor concentrations in transplantable tumours of the TG3 line, and half-solid circles those in transplantable tumours of the TG5 line.*

tumours is increased following oestrogen administration, i.e. is oestrogen-*sensitive*.

The R3230AC transplantable mammary tumour is also not *ovary-dependent* yet is sensitive to oestrogen [18] and similarly contains low levels of oestrogen receptor activity [14]. According to Sluyser and Van Nie [11] transplantable mammary tumours may change from "dependent" to "responsive" to "independent" tumours, in association with diminishing receptor levels upon successive transplantations. Although the data presented here was too small to show such significant differences in receptor levels between generations, it is our impression that the receptor levels in our TG3 and TG5 lines are also gradually diminishing with succeeding transplantations.

In the transplantable tumours studied here (Table 1), as in the R3230AC tumour [19] available receptor levels were undiminished by ovariectomy, though this finding could be the resultant of a decrease in *total* receptor sites and an increase in the number of available sites, upon removal of endogenous hormones. In contrast, the level of available receptor activity falls significantly after ovariectomy in most DMBA-induced tumours [6, 20, 21] where both receptor synthesis and general anabolism may depend on the presence of the ovary. This fall in available receptor level in DMBA-induced tumours after ovariectomy, which we [6] and others (20, 21) have previously reported, was not seen in present work [Fig. 3, group (a) vs group (c)], probably because the receptor levels were measured in tumours from exsanguinated, ovariectomised rats (group a) but non-exsanguinated, intact rats (group c). The presence of a higher blood content in the latter type of tumour may account for our finding of receptor levels lower than those which we found previously [6] for DMBA-induced tumours from intact rats, and for the lack of difference between the values for tumours from intact and ovariectomised rats, since blood plasma can markedly suppress the level of receptor activity detected [22].

Early work with DMBA-induced mammary tumours implied very clear differences in magnitude of receptor levels between dependent and independent tumours of the rat [1–5]. More recently, Costlow, Buschow, Reichert and McGuire [23] also found clearly higher levels of both oestrogen and prolactin receptor activity in an ovary-dependent subline (MD) of a transplantable mammary tumour (MTW9) than in an ovary-independent subline (MA). In the present work, significantly lower *mean* receptor concentrations were found in the TG3 and TG5 tumour lines (ovary-independent) than in

DMBA-induced tumours (80–100% ovary-dependent) in each of three endocrine states. However, there was a considerable overlap in receptor values between the two types of tumour in intact animals, or in animals treated by ovariectomy only.

This finding of a less striking difference than was first proposed is in line with the views of Mobbs and Johnson [15], Boylan and Wittliff [16] and DeSombre, Kledzik, Marshall and Meites [13]. In the latter work, in particular, for both oestrogen receptors and prolactin receptors, there was a clear overlap between the concentrations found in dependent and independent mammary tumours. It is to be noted, however, that strictly only measurements on *biopsy* specimens taken prior to endocrine manipulation relate to the hormonal sensitivity at that time, and are comparable to the usual clinical situation where for prognostic use, biopsy material is taken *before* endocrine therapy.

For technical reasons (low incidence of DMBA-induced tumours in inbred rats, low transplant-acceptance rate in random-bred rats), the comparisons made here between DMBA-induced and transplantable tumours are complicated by the fact that the tumours were generated in rats of different strains. These strains of rat have different basal levels of prolactin (24, 25 and c.f. values in Table 1 with those in reference [6]), a hormone which has previously been shown to modulate tumour receptor levels [20, 21]. Thus it is possible that the lower receptor levels detected in transplanted tumours may be related to the lower levels of this hormone which circulate in our inbred rats, rather than to a lesser degree of hormonal sensitivity. This possibility is currently being investigated.

Irrespective of the origin of this overlap in receptor concentrations between dependent and independent rat mammary tumours (e.g. effect of strain, hormonal levels or variation in degree of hormonal sensitivity) extrapolation of these findings to the clinical situation would suggest that predictions based on oestrogen receptor concentrations should be made with caution. Thus at the present time, only "receptor-negative" tumours, (i.e. those with receptor levels clearly below the overlap range) can be accurately predicted to fail to respond to endocrine therapy.

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The Gross and Cellular Response of a Rat Mammary Tumour to Single Doses of Cyclophosphamide*

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Abstract—*The response of a transplanted mammary carcinoma of Wistar rats, to single intraperitoneal doses of cyclophosphamide* (25–250 mg kg^{-1}) *has been assayed by measurement of; delay in growth of the tumour between* 9 *and* 25 mm; *cell survival* in vitro, 3 *and* 24 hr *after* in situ *treatment; tritiated thymidine autoradiography and Feulgen microdensitometry of histological material. All three methods of assay indicate that at* 9 mm *diameter the tumour contains a subpopulation of malignant cells relatively resistant to cyclophosphamide doses in excess of* 50 mg kg^{-1}. *The* in vitro *cell survival assay suggests that these cells comprise* 40–50% *of the clonogenic population and are capable of considerable* in situ *repair of potentially lethal damage within* 24 hr *of treatment. Cellular repopulation from this resistant component, after* 150 mg kg^{-1} *of drug, is delayed for* 6 *or more days and restoration to near pre-treatment status only achieved by* 10 days, *in this rapidly-growing tumour.*

INTRODUCTION

MOST drugs used routinely in cancer chemotherapy function by interfering with processes of cell multiplication [1]. Thus in comparing drug effects on e.g. a lymphoma cell population in rapid exponential growth with an unstimulated normal renewal tissue with relatively few cells in the replication cycle, the therapeutic advantage may be marked [2]. However, solid tumours in animals and humans can have 85% or more of their cells out of cycle [3] and these may be less susceptible to the action of cytotoxic drugs. If such cells are preferentially spared, then their therapeutic importance depends upon their number and capacity to re-enter cycle. This is known to occur in irradiated tumours [4], although the transition may not be rapid [5].

We have examined the response of an experimental solid tumour, known to contain a population of non-cycling cells [6], to cyclophosphamide (CP) whose cytotoxicity is held to be proliferation-dependent, but whose action is not markedly cell-cycle-phase specific [1]. In this study, the gross response to single doses of the agent has been defined and related to cellular survival and changes in cellular kinetics of the treated tumour.

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MATERIAL AND METHODS

1. *Animals and tumour*

Male and female John's strain Wistar rats were used, at a body weight of 150–200 g. Animals were kept under a 12 hr light (06.00–18.00), 12 hr dark (18.00–06.00) regimen and provided with food and water *ad lib.* Subcutaneous experimental tumours were prepared by implantation in the flank with an isogeneic mammary carcinoma (LMC_1) of spontaneous origin. Details of methods of transplantation and subsequent macroscopic and cellular growth have been reported elsewhere [7]. The present study employed tumours of the 37th to 40th transplant generations, rats being assigned randomly to treatment groups when the tumour reached a mean diameter of 9 ± 1 mm. Thereupon CP ("Endoxana"; Ward Blenkinsop, Bracknell) was injected intraperitoneally (i.p.) in 0.9% saline, as single doses of 25–250 mg kg^{-1} of body-weight. All injections were given between 11.00 and 12.00 hr.

2. *Assays*

The gross response of the tumour to CP was measured in terms of changes in tumour volume with time after treatment. These were computed from daily, caliper measurements of three mutually perpendicular diameters of each tumour. From these data, tumour response was de-

termined by the method of Thomlinson and Craddock [8] i.e. by plotting the delay in growth of the tumour between 9 mm (370 mm^3) and 25 mm (8000 mm^3) relative to untreated controls, as a function of CP dose.

The survival of clonogenic LMC_1 tumour cells *in situ* after exposure of 9 mm diameter tumour to CP was assayed *in vitro* by a method modified after that of Reinhold [9], as described by Moore [7]. Clonogenic cells were defined as those which, after 10 days' incubation at 37°C, had given rise to colonies of 50 or more cells.

The kinetic status of the overall cell population of LMC_1 tumours treated with CP was measured by tritiated thymidine (^{3}H-TdR) autoradiography and Feulgen microdensitometry; details of both methods have been reported elsewhere [6, 10]. Tumour-bearing rats, which had received doses of CP 1 hr to 10 days earlier, were injected i.p. with ^{3}H-TdR (0.5 μCi g^{-1} of body-weight) at 12.00–13.00 hr and killed 1 hr later. Autoradiographs of equatorial sections of tumours were prepared for determination of Labelling Index (LI), Mitotic Index (MI) and Cellular Density (CD; i.e. the number of cytologically-intact tumour nuclei per 100 × 100 μm microscope field). A minimum of 2000 nuclei were scored in each section and 4–5 sections, from different tumours, in each treatment group. For Feulgen microdensitometry, an imprint was made onto a microscope slide of the freshly-cut faces of the bisected tumours later used in preparing autoradiographs. A smear was also made of peripheral blood of the tumour host. After fixation and staining, measurements were made on a Vickers M85 integrating microdensitometer, small lymphocytes of the peripheral blood being used to determine the DNA content of normal diploid, non-proliferative cell nuclei. For each treatment group, 600 tumour nuclei and 50 lymphocytes were scanned at random, the material being taken from 2 tumours/hosts.

RESULTS

1. *Tumour growth delay*

Inhibition of the growth of LMC_1 tumours in treated rats was CP-dose dependent (Fig. 1). Only for the largest dose tolerated by female rats (250 mg kg^{-1}) did regression below treatment size occur. For tumours regrowing after treatment, the overall volume doubling time was not significantly higher than in untreated controls and did not vary with dose of CP. The time taken for the tumour to reach a mean diameter of 25 mm (14.1 ± 0.1 days) was the same in untreated male and female rats and overall this was true for the tumours in CP-treated rats. Therefore, tumour data from both sexes of host were used to establish the CP dose, tumour response curve. Delay in growth of the tumour was not a linear function of dose (Fig. 2). Between 25 and 75 mg kg^{-1}, an additional delay of 1 day was attained with each 5 mg kg^{-1} increment in dose. Between 75 mg kg^{-1} and the maximally-tolerated dose, a 25 mg kg^{-1} increment was required for the same effect.

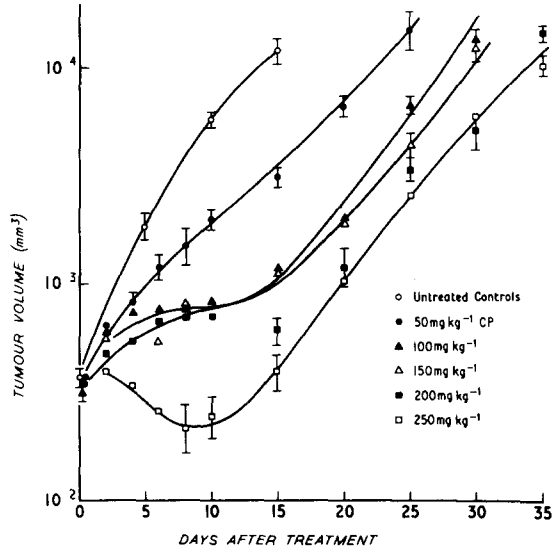

Fig. 1. Growth response of LMC_1 to CP. Mean ± 1 S.E. of data for 4–21 female rats per treatment group. Mean volume doubling time of untreated controls 4 ± 1 days.

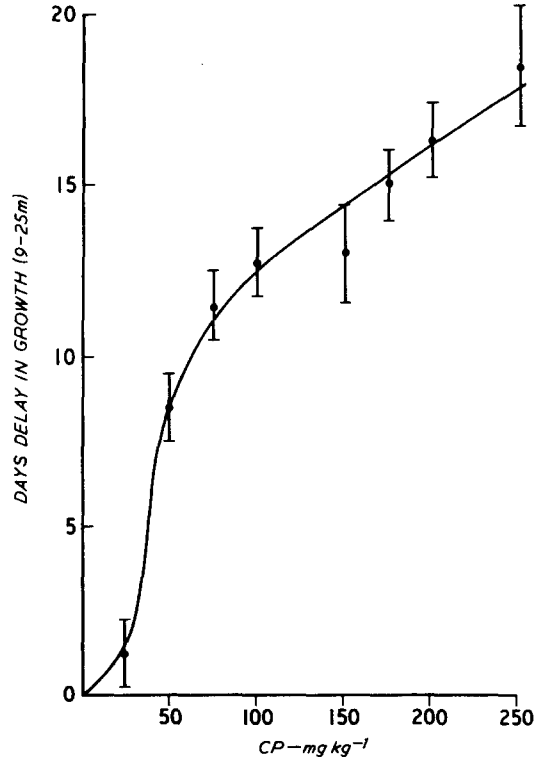

Fig. 2. Growth delay of LMC_1 after CP. Pooled data for male and female rats, 13–30 per dose-point. Mean delays ± 2 S.E.

2. *Survival of clonogenic cells*

The biological half-life of CP and its toxic metabolites in rats has been estimated to be 1–2 hr [11]. Assay for survival of LMC_1 cells was therefore carried out 3 hr after injection of the drug, when the cells should have been subjected to much of the integrated dose. However, cells *in situ* may be able to repair potentially lethal damage (PLD) due to CP [12]. A second assay was therefore carried out 24 hr after treatment, since if LMC_1 cells did so repair, results might be expected to be more closely comparable with those for the *in situ* end-point of growth delay. Within the range of CP doses used, 50–250 mg kg^{-1}, survival of clonogenic cells decreased as a logarithmic function of dose for both intervals (Fig. 3). However, a dose-dependent recovery of clonogenic capacity occurred between the two times, giving D_0 values for curves fitted to the two sets of data of 40 ± 5 mg kg^{-1} at 3 hr, 58 ± 3 mg kg^{-1} at 24 hr. In neither case did the best fit to the experimental data extrapolate to 100 % survival at zero dose, the intercept at 3 hr being 0.18 ± 0.06 and that for 24 hr, 0.44 ± 0.05. In both cases, the difference from unity was highly significant ($P < 0.001$).

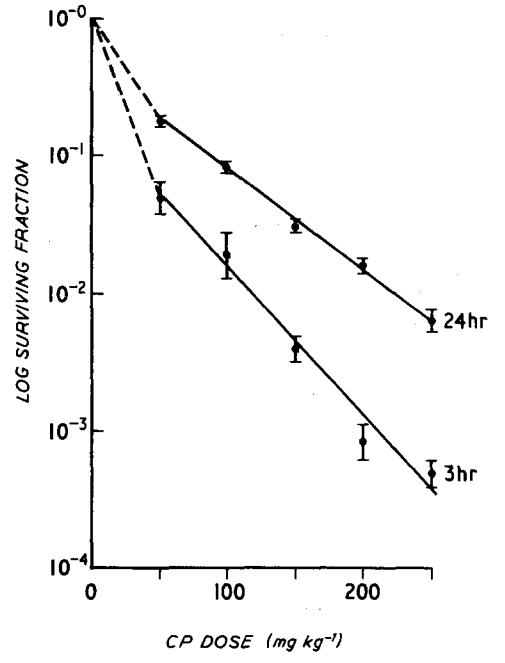

Fig. 3. In vitro *survival of* LMC_1 *cells after CP* in situ. *Mean surviving fractions* $\pm$ 1 *S.E. for replicate platings of cells from single tumours. It is assumed that the dose–response curves have no threshold* [12] *and are exponential for doses* < 50 mg kg^{-1}.

3. *Cellular kinetics after CP treatment*

The time dependence of kinetic changes in tumours of treated rats was examined following a dose of 150 mg kg^{-1}, this dose being approximately at the mid-point of the "resistant" component of the curve for growth delay (Fig. 2). It is evident from both the microdensitometric and ^{3}H-TdR data that cellular kinetics were perturbed for several days (Fig. 4, a and b). The frequency distribution of nuclear DNA content indicates that, in the first two days after treatment of this predominantly tetraploid tumour, the G_1 components were markedly depleted with a commensurate increase in the frequency of S and/or G_2 nuclei. Thus LI remained high during this time, but MI fell almost to zero. Although tumour volume increased by about 50% in these first two days (Fig. 1), the cellularity of the tumour fell by approximately 40% (Fig. 4, b). It is likely then, in the presence of the mitotic block, that the gross volume increase was largely due to changes in cell volume associated with the higher modal DNA content of the cell nuclei. Over the succeeding 4 days, LI was depressed, suggesting that a large proportion of the cells present at treatment had by now accumulated in G_2. Only at 10 days did the microdensitometric and ^{3}H-TdR data indicate that the tumour was regaining a kinetic state characteristic of the untreated condition.

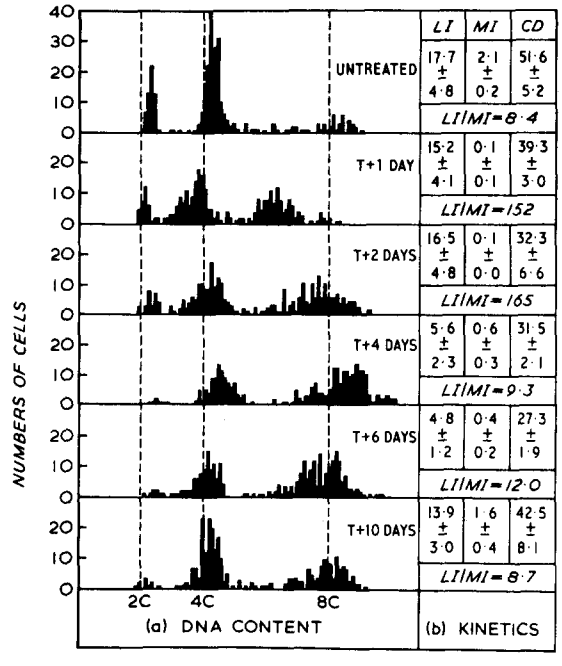

Fig. 4.a. *Nuclear DNA content of* LMC_1 *cells after* 150 mg kg^{-1} *of CP* in situ. *2c and higher integer values of DNA content established by comparison with small lymphocyte nuclei.*

b. *Corresponding values for LI, MI and CD measured from histological sections of* 9 mm LMC_1 *tumours treated with* 150 mg kg^{1} *CP. Mean values* (%) $\pm$ 1 *S.E. Median cell cycle time for untreated tumours* 18 hr [6].

To determine if the patterns of response to graded doses of CP revealed by growth delay and clonogenic cell survival were reflected in the kinetic behaviour of the cells, a further ^{3}H-TdR experiment was carried out. This assay was complicated by the early perturbations of phase relationships; the first time at which LI/MI ($\sim$Ts/Tm) was restored to pre-treatment value was 4 days (Fig. 4, b). Accordingly, rats bearing 9 mm tumours received single doses of 50–200 mg kg^{-1} CP and 4 days later were injected with ^{3}H-TdR and killed at 1 hr. From autoradiographs of tumour sections, LI, MI and CD were plotted as a logarithmic function of dose (Fig. 5). The values of the three parameters declined rapidly with increasing dose up to about 100 mg kg^{-1}. Doses greater than this had little additional effect on ^{3}H-TdR labelling or cellularity of the tumour and a reduced effect on the frequency of mitoses.

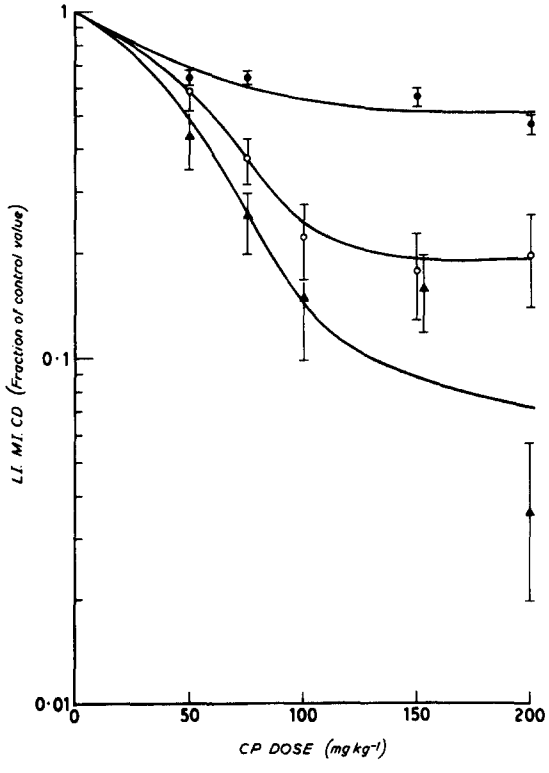

Fig. 5. Cell kinetic response of LMC$_1$ to graded doses of CP, measured 4 days after treatment. Values of LI ○, MI ▲, CD ●, relative to untreated controls, ± 1 S.E.

DISCUSSION

The gross response of the LMC$_1$ tumour to CP has been measured by tumour growth delay, an end-point with which much information has previously been obtained on the response of experimental tumours to irradiation [8, 13]. From such studies it is seen that where all the cells of a tumour are of equal apparent sensitivity to radiation, e.g. when the tumour is made "anoxic", the dose–delay curve rises smoothly with increasing slope. Conversely, where the aerobic tumour contains two subpopulations of different sensitivities, a biphasic delay curve results, with reduced slope at higher doses. This was the form of the delay curve for LMC$_1$ tumours treated with CP (Fig. 2), indicating by analogy that the tumour normally contains cells that are preferentially spared by GP.

The cells that are important in tumour regrowth following a period of delay are ultimately those capable of repeated division. The *in vitro* survival of clonogenic cells in LMC$_1$ after CP depended upon the time of assay (Fig. 3), in a manner consistent with the repair of PLD *in situ* [12]. It is usually held that in tumours such repair is predominantly of slowly-cycling or non-cycling cells [14]. If this is true for the CP-resistant component of cells in LMC$_1$, then extrapolation of the 24 hr-survival curve back to the ordinate (Fig. 3) indicates that these constitute 44 ± 5% of the cell population in 9 mm tumours. This value compares favourably with that for the non-cycling population of 40% estimated previously by ^{3}H-TdR analysis [6].

Any attempt to describe the subsequent fate of the therapeutically important, surviving clonogenic cells in terms of cellular kinetics of the treated tumour is complicated by the fact that kinetic analyses usually sample not only from a population of surviving clonogenic, but also proliferative though non-clonogenic cells [15]. Thus in the first 2 days after 150 mg kg^{-1} CP, LI remained high despite a mitotic block (Fig. 4, b). Our interpretation of the subsequent sharp fall in LI is that the majority of cells in S up to this time were "terminal" and by 4 days had accumulated as non-proliferative cells in G$_2$ (Fig. 4, a). The low LI and MI between 4 and 6 days would thus be characteristic of a surviving population in or entering cycle no less than 2 days after drug treatment, but which as yet represented only a small proportion of an overall population containing many terminal cells. For these surviving cells the ratio LI/MI was approximately that of the untreated tumour.

If at 4 days these labelled and mitotic cells represented the surviving "clonogenic" population, then the values of the kinetic parameters at this time following different doses of drug should indicate their relative survival *in situ*. These remained virtually unchanged with doses of CP in excess of 75–100 mg kg^{-1} (Fig. 5). In addition, the change in value with time after 4 days in LI, MI and CD would reflect the rate of cell repopulation within the tumour. After 150 mg kg^{-1} CP, little repopulation had occurred by 6

days, most thereafter (Fig. 4). In these circumstances it might be expected that a further dose of CP would be more effective at longer rather than shorter intervals, i.e. as cells re-enter cycle, particularly if this permitted a reduced response in therapeutically-limiting normal tissues. This hypothesis is being tested experimentally.

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Letter to the Editor

Efficacy of Combined Interferon Cyclophosphamide Therapy after Diagnosis of Lymphoma in AKR Mice*

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ALTHOUGH the spontaneous lymphoma of AKR mice has been considered a useful model for the evaluation of chemotherapeutic drugs [1–8], very few substances are effective in delaying death. Frei *et al.* reported that only 4 of 27 compounds useful in cancer chemotherapy increased by 100% or more the mean survival time of AKR mice after diagnosis of lymphoma [8]. Of these, cyclophosphamide was the most effective drug [3, 4, 8]. Interferon may also be considered an effective drug since daily treatment of AKR mice increased average survival time by approximately 100% after diagnosis of lymphoma [9]. Since experimental and clinical results have shown that combination drug therapy is often superior to single drug therapy, we have investigated the effect of combination interferon–cyclophosphamide therapy in this experimental system. Chirigos and Pearson reported the striking efficacy of interferon and BCNU in the treatment of mice injected with LSTRA leukemia cells [10]. To our knowledge this is the only published report of combined interferon-antitumor drug therapy in an experimental tumor system. In view of the increasing interest in the use of interferon in the treatment of patients with various forms of neoplasia [11–14] it would seem important to determine to what extent, based on animal experimentation, combined therapy can be envisaged in man.

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Female AKR mice more than 6 months of age were purchased from Jackson Laboratories, Bar Harbor, ME. All mice were examined daily for enlarged inguinal, axillary and submaxillary lymph nodes, enlarged spleen and signs of respiratory distress secondary to an enlarged thymus. Mice were included in the study only when they had definite peripheral lymphadenopathy *and* splenomegaly (+, on a scale of 0 to +++). A few of these mice also had signs of some respiratory distress. Mice were *not* included if they had only minimal adenopathy, only peripheral adenopathy, only splenomegaly, or only signs of respiratory distress. Upon diagnosis of lymphoma each mouse was marked and treated or left untreated according to a prearranged plan. Each mouse was then weighed daily and the evolution of clinical signs of disease was recorded. All dead mice were autopsied. There were 4 groups of mice: untreated; treated with one intraperitoneal (i.p.) injection of cyclophosphamide (Lucien-Collombes Laboratories, France) (150 mg/kg); one injection i.p. of cyclophosphamide followed 3 days later by daily administration i.p. of 0.25 ml of control preparation; one injection of cyclophosphamide followed 3 days later by daily administration of 0.25 ml i.p. of a mouse C-243 cell interferon preparation titering 3.2×10^{-6} (specific activity of approximately 2.8×10^6 to 1.2×10^7 units/mg protein). Techniques of production, semipurification and assay of interferon and control preparations have been previously reported [15]. (Interferon units refer to interferon reference units.) In all instances cyclophosphamide induced a rapid (1–3 days) decrease in

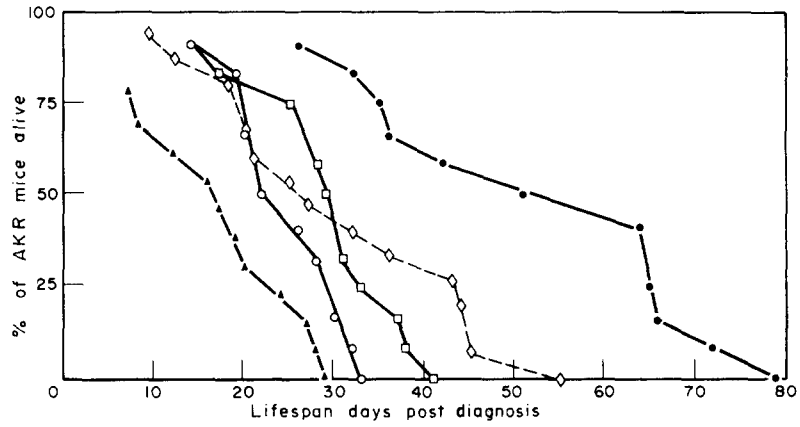

Fig. 1. Effect of interferon–cyclophosphamide therapy on survival of AKR mice after diagnosis of lymphoma. Untreated ▲——▲; cyclophosphamide × 1 □——□; cyclophosphamide × 1 followed by injections of control preparations ○——○; cyclophosphamide × 1 followed by injections of mouse interferon ●——●; results of a previous experiment [9] with interferon alone ◇——◇ see text.

Table 1. Effect of interferon–cyclophosphamide therapy on survival of AKR mice after diagnosis of lymphoma

Treatment	No. of mice	Mean survival ± S.E. (days)		Range of survival time (days) Confidence interval (0.95)
None	13	17.0 ± 2.3		(12.5–21.5)
Cyclophosphamide ① × 1	12	29.3 ± 2.3	*	(24.8–33.8)
		N.S.		
Cyclophosphamide × 1 Control preparation q.d.	12	24.7 ± 1.7	†	(21.3–28.1)
Cyclophosphamide × 1 Interferon 3.2×10^6 q.d.	12	52.7 ± 5.2		(42.6–62.9)

N.S.: not significant.
*: $P < 0.01$.
†: $P < 0.001$.
q.d.: Each day.
①: Cyclophosphamide 150 mg/kg.

signs of lymphoma (i.e. enlarged inguinal, axillary and submaxillary lymph nodes, enlarged spleen and thymus).

As can be seen from Fig. 1 and Table 1, a single injection of cyclophosphamide increased significantly the survival time of AKR mice after diagnosis of lymphoma from a mean of 17 days to 29 days. No significant difference was observed between mice injected with cyclophosphamide alone and those injected with cyclophosphamide followed by daily injections of a control preparation. In contrast, treatment of mice with one injection of cyclophosphamide followed by daily administration of mouse interferon increased survival time by approximately 200% (Fig. 1, Table 1).*

Because of the limited amount of potent interferon preparations available we did not include in this experiment a group of mice receiving interferon alone, but have plotted on Fig. 1 in dashed line (◇— — —◇) the results previously obtained in this system with interferon [9]. (Comparison of the survival time after diagnosis of lymphoma of untreated AKR mice in the previous published [9] and current experiments showed no significant difference.) The previously observed increase in survival time for interferon treated AKR mice [9] Fig. 1 and the increased survival time for cyclophosphamide treated mice in this experiment, suggest that the therapeutic effects of interferon and cyclophosphamide may be additive.

Although cyclophosphamide treatment considerably reduced the tumor burden, such

*Attempts in a subsequent experiment to investigate the effect of daily interferon treatment with repeated doses of cyclophosphamide [8] could not be pursued due to mortality in mice receiving several injections of cyclophosphamide.

treatment did not eliminate all tumor cells and mice eventually died with lymphoma. Our previous results [16–18] have shown that interferon treatment was most effective when the tumor burden was low. Thus the experiment reported above, although performed with relatively few mice, showed that after reduction of tumor load with cyclophosphamide interferon treatment increased survival time by approximately 200%. These results and those of Chirigos and Pearson [10] may have some relevance to the treatment of patients with neoplasia since they suggest that interferon therapy may be of particular benefit when administered in combination with anti-tumor chemotherapy.

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Perspectives in Cancer Research

Increased Selectivity of Drugs by Linking to Carriers

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THE THERAPEUTIC effectiveness of antitumoral, and of antiprotozoal drugs is very much restricted by their lack of selectivity towards their respective targets. Since the biochemical and metabolic properties of the normal host cells and the pathological cells are very small if not inexistent, it is very difficult to modify the chemical structure of a drug in such a way that it acquires target recognition abilities while keeping its pharmacological properties. It is therefore not surprising that many efforts have been spent since several years to increase the selectivity of drugs by association or linkage to a carrier molecule that would carry the active compounds to the target cells and prevent their interaction with normal cells.

Without being exhaustive we can enumerate:

(a) the association of antitumoral drugs to, antibodies [1–9], proteins [10–14], DNA [15–16], liposomes [17–25] and to hormones [26, 27].

(b) the linking of antiviral compounds to dipeptides [27], proteins [28] and synthetic polymers [29].

(c) the binding of antiprotozoal drugs to DNA [30].

(d) the inclusion of antiinflammatory agents, antibodies, enzymes and toxins in liposomes [31].

In this paper we will review the "drug-carrier concept" with special emphasis on its implications at the cellular level. We will also try to draw some guidelines for further research in this field.

We will define a "carrier" as a molecule or an assembly of molecules, the major characteristic of which is that it is unable, as such or bound to a drug, to cross the plasma membrane of eukaryotic cells by permeation or active transport.

ADVANTAGES OF DRUG TO CARRIER LINKING

As mentioned above the major advantage sought for by the use of drug-carriers is a higher selectivity of action. The carrier can induce the drug to act selectively in two ways. The carrier–drug complex can, first of all, be selectively activated in the vicinity of the target cells by factors released from these cells or present at their surface. The carrier can on the other hand recognize selectively the target cell surface and allow the drug to act either at the surface or intracellularly after uptake of the carrier–drug complex.

The association of a drug with a carrier can also modify its mechanism of intracellular penetration and promote the cellular uptake of drugs that cannot enter cells because of a native or acquired membrane impermeability.

The rate and degree of excretion as well as the intratissular distribution of the carrier-linked drug will also be modified at a variable extent according to the size and the nature of the carrier. As a consequence the pharmacokinetic properties of the drug could be modified and result in higher as well as longer sustained plasma and extracellular levels of the active compound. This can induce secondarily a higher therapeutic effectiveness.

LIMITATIONS OF DRUG TO CARRIER-LINKING

Since the adsorption of a non-permeant carrier through the gastro intestinal tract, skin or mucosa, seems highly improbable, the use of carrier linked drugs will probably preclude any oral or topical administration.

Restriction will also be imposed on the parenteral use of drug–carrier combinations according to the anatomical and tissular localization of the target. The tissular distribution of the drug-carrier will indeed be restricted by the ability of the carrier to cross the various types of capillary membranes that separate the site of injection and the blood stream from the targets. This will depend very much on the physicochemical properties and the molecular size of the carrier as well as on the type of capillaries involved. It is indeed very well known that the structure of capillary membranes varies very much from one tissue and organ to another. Capillaries of liver, spleen and bone marrow will very probably not constitute a great obstacle even to the largest of the possible carriers that are liposomes. These capillaries are indeed characterized by an incomplete basal membrane and by non contiguous endothelial cells. If one considers however, other tissues like endocrine glands, intestine and muscle, the situation becomes more complex since the capillaries of these tissues are characterized by a complete basal membrane and a continuous lining of endothelial cells. Macromolecules and liposomes will certainly have great difficulties in crossing these membranes and in reaching targets localized across the capillary wall and the obstacle will of course be the greatest in the central nervous system. We have however to consider that in many pathological states, like tumours or infections, the capillaries can be modified and their structure as well as their permeability properties altered.

In preparing drug-carrier complexes, great care should be taken in order to avoid any direct or indirect toxic effects induced by the carrier itself. The carrier should be non toxic, non immunogenic (as such or in combination with the drug) and biodegradable. This latter property is important in order to avoid cellular overloading and its toxic consequences as already observed after clinical use of poorly or non degradable molecules like polyvvynylpyrrolidone.

CARRIERS AND DRUG-CARRIER LINKS

In order to discuss the different possible carriers as well as the requirements for the drug to carrier links we will have to distinguish first, drugs acting exatracellularly from those acting on the host cells. In this latter category we will then discuss in more detail the various possible interactions between cells and the drug-carrier complexes.

(1) *Extracellular site of action*

Amongst the drugs with an extracellular site of action, that are liable to benefit of a carrier linking we count: (a) drugs acting on extracellular protozoa, parasites and bacteria; (b) enzymes like asparaginase, uricase; (c) compounds that counteract the deleterious effects of enzymes released in the extracellular space during pathological processes like inflammation, rheumatoid arthritis or shock.

The carriers should in this case (a) have a molecular size that prevents them from crossing the endothelia and capillaries lining the various vascular spaces and body cavities; (b) not to be captured at a great extent by the macrophages and the reticulo endothelial system; (c) be small enough to be excreted by the kidney in order that all the compounds that do not reach the targets can be eliminated in an inactive form.

If the link with the carrier does not interfere with the activity of the drug the main advantage of the carrier complex will be to provide the drug with different pharmacokinetic properties. The drug could in this way be maintained for a longer time and at a higher concentration in the blood stream or various body compartments like the peritoneum, pleural cavity, joint cavity or cerebrospinal fluid. The inclusion in a suitable carrier like a liposome can also decrease the immunogenicity of the active compound and decrease its reactivity with free or cell-bound antibodies [19, 32].

Some degree of selectivity can be conferred to drugs of this category if the carrier linked drug is inactive and can be released from the carrier in an active form by, or in the vicinity of the extracellular target. Such a selectivity could for example be obtained in the treatment of extracellular infections by protozoa, or parasites, that secrete exoenzymes like collagenase, polysaccharidase or proteases. If the link between the drug and the carrier is susceptible to hydrolysis by one of these enzymes, the drug will be released and activated specifically in the vicinity of the extracellular targets. In the case of infections by protozoa or parasites the carrier drug complex can also be taken up by endocytosis or ingested and the drug released inside the lysosomal or digestive system of the parasite. The selectivity of the drug complex will depend in this case on the avidity of the infectious agent for the carrier molecule and on the digestibility of the carrier and the carrier drug link.

(2) *Intracellular site of action*

The selectivity of a carrier complex formed with a drug acting intracellularly can be modulated by: (a) a selective release in the

vicinity or at the surface of the target cell; (b) a selective uptake by the target cell.

(*a*) *Extracellular release.* A selective release of active compounds from suitable carriers could be achieved in at least two pathological situations involving the selective release of hydrolytic enzymes by cells involved in the pathological process (Fig. 1). In inflammatory and rheumatoid diseases, proteases and collagenases have indeed been shown to be secreted in the extracellular space. The same could be true in some types of cancer cells that have been shown to excrete or possess at their cell surface proteolytic enzymes. The ideal carrier in this situation should: (a) not be endocytized by the reticulo endothelial system; (b) be small enough to be excreted by the kidney; (c) the drug to carrier link should be sensitive to one of the enzymes excreted selectively by the target pathological cells.

(*b*) *Intracellular release.* In this category of carrier-drug combinations the selectivity will be

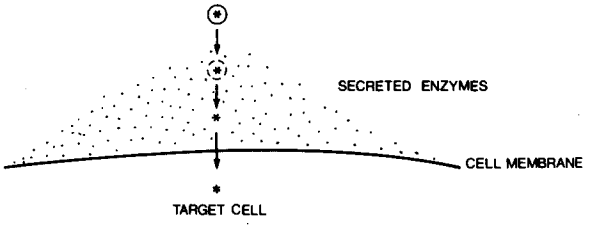

Fig. 1. *Drug-carrier complex activated in the extracellular space by enzymes released from the target cells. Symbols of Figs.* 1, 2 *and* 3: ⊛ *inactive drug-carrier complex;* ○ *carrier;* * *free and active drug.*

determined by a selective interaction between the carrier and the target cell surface. This interaction will then be followed by an intracellular penetration of the complex that can occur either by endocytosis or by transport through the cell membrane if the drug is included in a particular type of macromolecular vesicles.

UPTAKE BY ENDOCYTOSIS (LYSOSOMOTROPIC DRUG-CARRIER)

The most common mode of cellular penetration of a non-permeant carrier is endocytosis. During this process, the carrier-drug complex will reach the lysosomes after fusion of the endocytic vacuole with the lysosomes [33] (Fig. 2). Inside the lysosomes, the drug can be released and activated if the carrier or the link between the drug and the carrier is hydrolyzed by the enzymes or the acid pH prevailing inside the lysosomes. The drug itself should of course not be destroyed in the same conditions.

After release the drug can act inside the lysosomes themselves and in this category we find (1) antibiotics acting against intralysosomal bacteria or protozoa; (2) antiinflammatory or antirheumatoid compounds that are supposed to act on the lysosomal constituents themselves (chloroquine, gold salts, corticosteroids); (3) lysosomal enzymes that can be used in the treatment of lysosomal storage diseases; (4) agents that could destroy the lysosomal membrane, induce the intracytoplasmic release of lysosomal hydrolases and lead to the destruction of the cell.

A drug-carrier complex of this type will concentrate specifically the drug inside the cell compartment wherein it is supposed to act. It will also allow active compounds that are unable to cross the plasma membrane to reach the lysosomes.

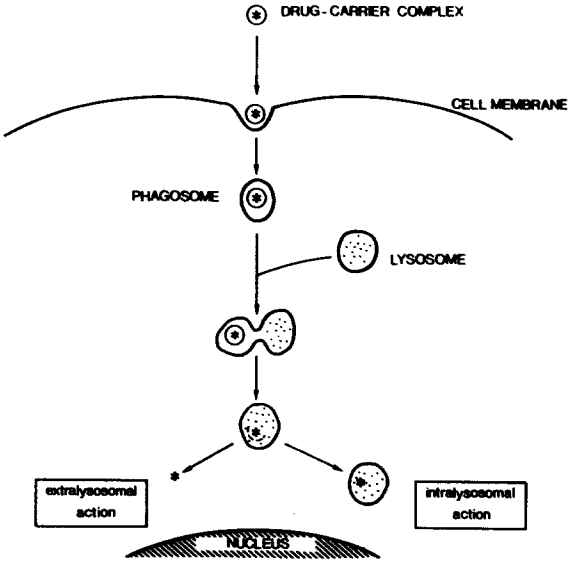

Fig. 2. *Mechanism of entry and mode of action of a drug-carrier complex taken up by endocytosis.*

The usefulness of a lysosomotropic carrier complex can be extended to drugs acting on extralysosomal compartments provided that the drugs are able to cross the lysosomal membranes.

Moreover, the selectivity of the lysosomotropic complexes will depend not only on the specific interaction of the carrier with the cells but also on the endocytic activity of the target cells.

UPTAKE THROUGH THE CELL MEMBRANE

It has been shown recently that unilamellar lipid vesicles can in some conditions, fuse with cellular membranes and release their content directly inside the cytoplasm [34, 35] (Fig. 3). This peculiar type of carrier could allow to introduce inside the cytoplasm (a) macromo-

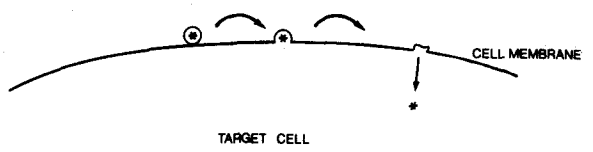

Fig. 3. Mechanism of entry of a drug included in a unilamellar lipid vesicle fusing with the cell membrane.

lecules that evade in this way the hydrolytic enzymes of lysosomes; (b) drugs that cannot permeate through the cellular or lysosomal membranes. When the technology of these vesicles will be mastered and their cell behaviour very clearly analyzed, the field of genetic engineering and enzyme replacement therapy will be wide open as well as the possibility of bypassing the cell membranes by any drug towards which these membranes are unpermeable.

CELL SELECTIVITY MEDIATED BY CARRIERS

If the ultimate goal of the carrier binding is to obtain drugs that recognize and interact specifically with the target cells it is important to consider that two intermediate levels of improved selectivity can be achieved by linking drugs to suitable if not optimal carriers.

All drugs, acting at the intracellular level, penetrate, after administration, into most of the cells of the body by permeation through the cell membrane. By linking these active compounds to a carrier, the tissular distribution and cell uptake will become more restricted than that of the free drug. Cells possessing low endocytic activities or separated from the site of administration by capillar membranes unpermeable to the carrier will remain out of reach. If the target cells do not fall in this category, the drug carrier will remain active and its side effects reduced.

A higher level of selectivity will be reached in the case of drugs linked to an endocytozable carrier if the target cells display a very high endocytic activity towards the carrier. In these conditions, the drug carrier will not only be less toxic but also more active if its rate of penetration and intracellular concentration is higher than if the active compound was given alone.

POSSIBLE CELL SELECTIVE CARRIERS

Except for the drug-carrier complexes acting or released extracellularly the selectivity of a carrier will depend on a specific interaction with the plasma membrane of the target cells. As possible cell selective carriers we can enumerate as follows:

(1) Antibodies directed against cell specific antigens.
(2) Peptidic hormones, that like insulin, vasopressin, thyrotropin, etc., interact with the surface membrane of some cell types.
(3) Viral envelopes or surface components responsible for the selective interaction of viruses and some cell types.
(4) Lectins.
(5) Selectively endocytized macromolecules like for example asialofetoproteins selectively taken up by hepatocytes.
(6) Polypeptidic fragments of toxin (diphtheria, ricin, abrin) responsible for the cellular fixation and penetration of the toxin.

The recognition and selectivity properties of carriers can on the other hand only come to their full expression if the carrier is not taken up in a random way by the cells with which the carrier comes in contact first after administration, namely endothelial cells and cells of the reticulo endothelial system.

In the case of lysosomotropic drug-carrier complexes, the carrier should, after reaction with the cell surface, induce its endocytosis by the cell. Very little is however known today about the factors that determine the fate of a macromolecule after its fixation to the cell surface. Using antibodies of various specificities and rat embryo fibroblasts in culture, we have shown in collaboration with Y. J. Schneider and P. Tulkens [36, 37] that the type of surface antigens with which antibodies react play an important role in determining the intracellular fate. Antibodies directed against lysosomal antigens react with the cell surface and are subsequently very quickly endocytized and concentrated inside the lysosomes. By contrast antibodies directed against plasma membrane antigens seem to remain associated for a very long period with the cell surface and are not accumulated at a great extent in the lysosomes. The fate of IgG molecules can also be modified by fixation of fluorescein isothiocyanate. While normal IgG are endocytized very slowly and at a very low extent by cells in culture, fluorescein labelled IgG are taken up very rapidly and specifically transferred inside the lysosomes.

A similar problem arises with regard to the unilamellar vesicles, the physicochemical properties of which probably determine their cellular uptake by endocytosis or their fusion with the cell membrane [35].

EXAMPLES OF DRUG-CARRIER COMPLEXES WITH INCREASED SELECTIVITY

To illustrate the modifications in selectivity that can be reached by linking drugs to carriers we will briefly summarize our results obtained in the field of cancer and trypanosomiasis chemotherapy by linking drugs to DNA.

Daunorubicin and adriamycin, very potent but very toxic antitumoral drugs, can be transformed into lysosomotropic drug-carrier complexes by linking to high molecular weight DNA. In experimental tumours it has been shown that daunorubicin-DNA and adriamycin-DNA are less toxic than the free drug. Heart [38], liver and kidney are protected at a very great extent against the toxic action of the drugs, this protection is lower in the case of bone marrow and absent with regard to the lymphoid tissues [39]. Although less toxic the DNA complexes are more active than the free drug in treatment of L1210 leukemia mice. Preliminary results in the treatment of human leukemia and various solid tumours indicate also that the cardiotoxicity of ADR and DNR could be reduced when given as DNA complexes while retaining their therapeutic effectiveness.

The lower toxicity of the DNA linked drugs is probably due to a decreased uptake of DNR and ADR by some normal cell types either because of their lower endocytic activity or because of their inaccessibility to macromolecules. The higher chemotherapeutic effectiveness can be explained by improved pharmacokinetic properties of the DNA complexes [40] and by the avidity and endocytic activity of the tumour cells towards DNA.

We have also shown that complexes formed between ethidium bromide and DNA have a lower toxicity than free ethidium bromide and a higher chemotherapic efficiency in the treatment of mice infected with Trypanosoma cruzi [30]. The increased efficiency of DNA-ethidium bromide can be due to a higher uptake of the complexes by the endocytizing parasites during their stay in the blood stream. It could also be explained by a selective concentration of the drug in the lysosomal system of the host cells that shelter the parasites during their intracellular stage. The first explanation is highly unlikely since DNA-ethidium bromide displays a lower chemotherapic efficiency against Trypanosoma brucei which are exclusively extracellular parasites.

CONCLUSIONS

A great deal of basic and applied research is still needed before the "drug-carrier" concept can be developed at the highest degree and provide highly selective drugs designed on the basis of their biochemical effects while making use of the enormous possibilities arising from the ever expanding field of cell biology.

Various molecules will have to be purified or synthetized in order to be screened as possible carriers.

(1) Plasma proteins.
(2) Cell specific antibodies.
(3) Nucleic acids.
(4) Peptidic hormones.
(5) Polysaccharides.
(6) Synthetic polypeptides (with various molecular weights and amino acid compositions).
(7) Lectins.
(8) Liposomes.
(9) Unilamellar lipid vesicles.
(10) Biodegradable microcapsules.

While screening these compounds special emphasis should be put on the analysis of the following properties: (1) cell selectivity; (2) interaction with cells; with special emphasis on their endocytosis or in the case of lipid vesicles on their fusion with plasma membrane; (3) uptake by the reticulo endothelial system; (4) passage through capillary barriers; (5) tissular distribution.

Finally techniques have to be developed that will allow drugs to be linked to macromolecules in such a way that they can be released from these carriers in an active form by hydrolysis of the drug-carrier link or by digestion of the carrier.

Although not new, the concept of vectoring drugs selectively to their target by carriers is becoming more and more liable to clinical application. The recent developments in cellular and molecular biology, as well as the progress achieved in organic chemistry will contribute decisively to the design of highly selective drug-carrier complexes so badly needed in the treatment of cancer, protozoal and degenerative diseases.

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Neuraminidase-like Effect of Vitamin A on Cell Surface*

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Abstract—*Control human lymphocytes and mouse Landscutz ascites tumour cells were treated* in vitro *with vitamin A alcohol. Vitamin A treatment produced changes in the cell wall which resulted in the release of sialic acid. This was similar to the effect observed when the cells were treated with the specific enzyme neuraminidase. Exposure of positive charges by vitamin A may lead to increased immunogenicity of allogeneic blast cells.*

INTRODUCTION

PRE-TREATMENT with vitamin A has been shown to enhance the therapeutic effectiveness of radiation and chemotherapeutic agents [1–3]. The vitamin exerts growth inhibitory effects on cultured murine leukaemic cells [4] and is associated with increased acid hydrolytic activities and increased tumour growth inhibition when given with cyclophosphamide [5].

Vitamin A can interact with and produce alterations of lysosomal membranes, resulting in the release of acid hydrolases [6–8] which may account for the increase in susceptibility of tumour cells to chemotherapeutic drugs [4]. However, changes in surface character of the cells, such as charge, antigenic properties and recognition by macrophages are produced by vitamin A [1–3] and these effects may account for the increased tumour cell susceptibility to chemotherapeutic drugs [4, 5]. It is known that vitamin A affects cells due to its penetration of lipoprotein membranes followed by formation of a micellar configuration of membrane lipids. The specific orientation of vitamin A within the membrane leads to the formation of a highly reactive moiety which is involved in electron transfer in or on the membrane of subcellular organelles [10].

Because of these actions of vitamin A on membrane structures, Brandes *et al.* [9] examined the cell surface of malignant cells exposed to vitamin A and under similar conditions compared this with neuraminidase treatment which removes negatively charged sialic acid from the glycoprotein surface. He noted the effects of vitamin A on Ruthenium Red staining and positive and negative colloidal particle binding and observed the similarities in the effects produced, i.e. loss of surface coat material, reduction in negative surface charge and exposure of positive charge.

We decided to investigate whether the cell surface changes induced by treatment of cells *in vitro* with vitamin A results in the release of free sialic acid due to neuraminidase activity or as peptide bound sialic acid due to non-specific hydrolytic activity.

MATERIAL AND METHODS

Vitamin A alcohol was obtained from Sgma Chemical Co., St. Louis, MO, U.S.A. and dissolved in 0.1% ethanol. *Vibrio cholera* neuraminidase (500 U/ml) was obtained from Calciochem. Cells used were (1) mouse Landscutz ascites tumour cells and (2) human lymphocytes from control subjects.

Treatment of cells

Seven day tumour cells were washed twice in Tris buffered saline pH 7.4 (TBS). They were resuspended in this medium at a concentration of 1×10^7 cells/ml before use.

Lymphocytes were obtained from whole blood (in preservative free heparin) by gradient centrifugation using Ficoll Triosil. The lymphocyte rich layer was washed twice in Tris buffered saline pH 7.4 and was resuspended in this medium at a concentration of 1×10^7 cells/ml.

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Incubation

Cells were incubated with Vitamin A/ethanol in varying concentrations to give a final concentration of 10–200 μg vitamin A/0.1% of ethanol/10^7 cells/ml. Cells containing 0.1% ethanol were used as controls. *Vibrio cholera* neuraminidase (VCN) was added to the cells suspensions at a concentration of 20 U/1 $\times 10^7$ cells/ml. Cells containing an identical volume of TBS were used as controls. All incubations took place in a shaking water bath at 37°C for 3 hr.

Viability

Dead cell counts were carried out under phase constrast on cell suspension mixed with an equal volume of lissamine green. VCN treated cells were 80–85% viable and did not differ from TBS controls by more than 5%. Vitamin A/ethanol-treated cells were 70% viable as were control cells containing 0.1% ethanol. The cells were removed by centrifugation for 5 min, at 500 ***g*** and the supernatants retained for estimation of free and bound sialic acid. Free sialic acid was estimated using the method of Warren [11] on supernatants which had been dried by rotary evaporation at 60°C and redissolved in 0.2 ml distilled warer. Bound sialic acid was measured by estimating the increase in sialic acid release from supernatants after refluxing for 3 hr with equal volume 2N (HCl).

RESULTS

Results show that vitamin A had a marked effect on the cells as viewed under the microscope. After 3 hr incubation with vitamin A, the cell surface had become crenated and many intracellular black spots were visible. The mean amount of sialic acid released from control cells (TBS) was 59.6 ± 10.3 nM/10^9cells and from control cells (0.1% ethanol) was 58.28 ± 15.6 nM/10^9 cells. In both cases 15 samples were measured.

The minimum effective concentration of vitamin A was 50 μg/10^9 cells when the mean 83.1 ± 5.2 nM sialic acid/10^9 cells was obtained. However, optimum concentration was 100 μg vitamin A/10^9 cells, when 96.60 ± 10.53 nM sialic acid/10^9 was released. This shows that the mean sialic acid released by 100 μg vitamin A/10^9 cells is 38.3 nM ($P > 0.005$).

In comparison the mean sialic acid released in VCN-treated cells (20 U/ml) was 176 ± 30.9 nM/10^9 cells, so the mean sialic acid released by the enzyme was 116.4 nM/10^9 ($P < 0.005$). The amount of sialic acid released by vitamin A is therefore 33% of the possible release of free sialic acid as measured by the increase in sialic acid after acid hydrolysis averaged 12 nM/10^9 cells but is not a genuine value as hydrolysed control values were not significantly different so sialic acid released as a result of vitamin A treatment is free sialic acid.

When lymphocytes from pooled blood of 5 control subjects were incubated at 37°C with vitamin A or with VCN as in previous experiments the following results were obtained. The mean sialic acid released from control samples was 94.9 ± 44.07 nM/10^9 cells (42 samples). Addition of 100 μg vitamin A/10^9 cells resulted in sialic acid values of 160.1 ± 55.2 nM/10^9 cells (12 samples). Therefore, 100 μg vitamin A released an average sialic acid of 65.2 nM/10^9 cells. In comparison, addition of 20 U/ml of VCN resulted in mean sialic acid levels of 309 ± 99.2 nM/10^9 cells which gives an average release of 214.3 nM sialic acid/10^9 cells by VCN. In lymphocytes, vitamin A released 30% of the possible sialic acid released by VCN. The sialic acid released as a result of incubation of lymphocytes with vitamin A is unbound sialic acid, as no significant increase in sialic acid levels was apparent when the supernatant was hydrolysed by refluxing with equal volume 2N HCl.

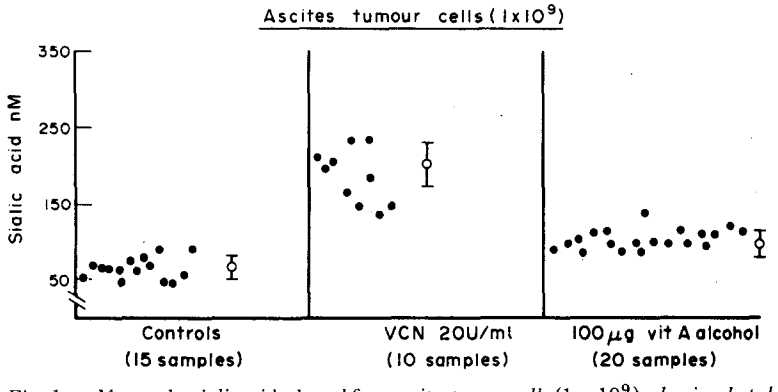

Fig. 1. Nanomoles sialic acid released from ascites tumour cells (1×10^9) *when incubated for* 3 hr *at* pH 7.6 *without additive (controls) and with VCN* (20 U/ml) *and vitamin A alcohol* (100 μg/ml) *respectively.*

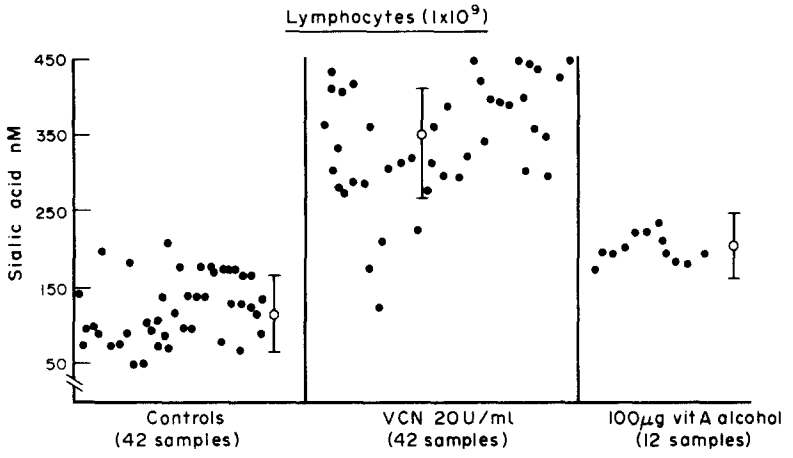

Fig. 2. Nanomoles sialic acid released from normal lymphocytes (1×10^9) *when incubated for* 3 hr *at* pH 7.6 *without additive (controls) and with VCN* (20 U/ml) *and vitamin A alcohol* (100 μg/ml) *respectively.*

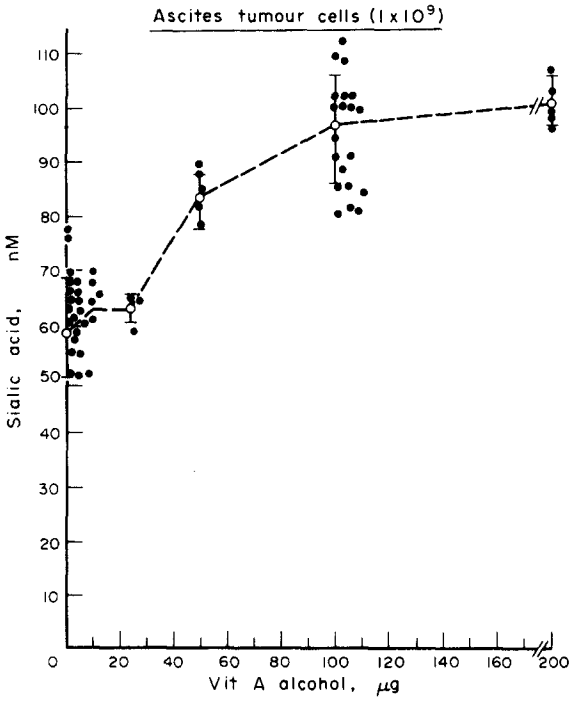

Fig. 3. Nanomoles sialic acid released from ascites tumour cells (1 $\times 10^9$) *when incubated for* 3 hr *at* pH 7.6 *with varying concentrations* (0–200 μg/ml) *vitamin A alcohol.*

DISCUSSION

The surface coat of the cells is affected by vitamin A [1–3] and the direct effect of vitamin A on the cell wall may account for the tumour cell increased susceptibility to chemotherapeutic drugs [4, 5]. These results show that cell surface changes induced by *in vitro* treatment with vitamin A result in the release of free sialic acid indicating an effect on cell surface similar to VCN which specifically cleaves the glycosidic link releasing terminal sialic acid.

Thus the reduction in negative surface change and exposure of positive charges on vitamin A treated malignant cells [4] is due at least in part to removal of negatively charged sialic acid from the cell surface. Holland and Bekesi [12] have shown strong evidence that pre-treatment of allogeneic myeloblasts with VCN increases the immunogenicity of these cells; VCN treated blasts when used in conjunction with chemotherapy have more than doubled the remission period in patients with acute myelogenous leukaemia. Whether vitamin A treated blasts would have a similar effect remains to be seen. Lundgren and Simmonds [13] using peripheral blood lymphocytes showed that VCN treated cells incubated with normal allogeneic lymphocytes in mixed lymphocyte culture (M.L.C.) more than doubled the rate of DNA synthesis compared to normal MLC response. We have found similar stimulation in MLC response when H^3 thymidine incorporation into 5-day-mixed cultures of lymphocutes and VCN-treated allogeneic blast cells. Work is now in progress to establish if immunogenicity of these blast cells is increased by incubation with Vitamin A.

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Transformation of 5-Aza-2′-deoxycytidine-^{3}H and Its Incorporation in Different Systems of Rapidly Proliferating Cells

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Abstract—*5-Aza-2′-deoxycytidine-*^{3}H *undergoes in Ehrlich ascitic cells phosphorylation and deamination resulting in the formation of new metabolites. In the acid-soluble pool of tetrahydrouridine-treated cells the level of deaminated analogue and of 5-azauracil formed by its phosphorolytic cleavage was substantially decreased. Both* in vivo *and in cell suspension* in vitro *5-aza-2′-deoxycytidine-*^{3}H *is incorporated into nucleic acids.*

In AKR mice with lymphatic leukemia the drug is preferentially incorporated into lymphatic tissues. While the blast infiltration of the liver results in a 3–4 fold increase of the uptake of the analogue the incorporation of thymidine-^{3}H *is enhanced 12–17 times to compare with the normal livers. Cytosine arabinoside depresses the incorporation of thymidine-*^{3}H *as well as 5-aza-2′-deoxycytidine-*^{3}H. *In regenerating rat livers thymidine, deoxycytidine but not 5-aza-2′-deoxycytidine are utilized with a greater efficiency than in the stationary liver. 5-Aza-2′-deoxycytidine is thus incorporated into rapidly proliferating cells while its uptake into lymphatic system is preferential.*

INTRODUCTION

5-AZAPYRIMIDINES represent a group of highly efficient cytostatics. Phase II and III studies have shown that 5-azacytidine is especially active in human acute myelogenous leukemia [1, 2] with an overall response rate of 36%. However, the clinical use of the drug is complicated by severe nausea and vomiting [1, 3]. Another compound of the group, 5-aza-2′-deoxycytidine, has also an impressive cytostatic activity [4, 5]. The drug is efficiently incorporated into nucleic acids in the lymphatic system [6, 7] and suppresses the cell growth in AKR mice with lymphatic leukemia, in P388 leukemia bearing mice and in L1210 leukemia [8].

The strong inhibitory effect of 5-aza-2′-deoxycytidine in microorganisms depends on its foregoing metabolic transformation, i.e. deamination and cleavage of deaminated nucleoside [9]. In higher organisms the analogue is also metabolized and several new compounds were detected in the urine of drug-treated mice [7]. 5-Aza-2′-deoxycytidine inhibits the phosphorylation of deoxycytidine and is incorporated into DNA [8]. Pretreatment of mice with the drug results in the inhibition of thymidine incorporation into spleen DNA in parallel with the decrease of thymidine and thymidylate kinase activities [7].

The aim of the present study was to follow the metabolic conversion of 5-aza-2′-deoxycytidine in tumor cells and to compare the incorporation and inhibitory effect of this antileukemic agent in different systems of rapidly proliferating cells.

Accepted 7 June 1977.

MATERIAL AND METHODS

Chemicals

5-Azacytidine, 5-aza-2′-deoxycytidine and cytosine arabinoside were prepared in the Department of Organic Synthesis of this Institute. Tritiated 5-aza-2′-deoxycytidine (19.5 Ci/mmole) was prepared by Dr. B. Černý, Isotope Laboratory, Prague-Krč. Thymidine (methyl-^{3}H) (8.1 Ci/mmole) and 2′-deoxycytidine-2-^{14}C (44 mCi/mmole) were provided by the Institute for Research, Production and Uses of Radioisotopes, Prague. Tetrahydrouridine was a gift from Dr. G. L. Neil, The Upjon Company, Kalamazoo.

Animals

Groups of 10 female mice (strain H, 25–28g) kept under standard laboratory conditions were transplanted i.p. 10^7 Ehrlich ascitic cells. The animals were used for the experiments 9 days later. Groups of 6 inbred AKR female mice were transplanted s.c. 2.10^7 leukemic cells and 7 days later were injected with the labelled compounds (maximal vol 0.2 ml, 2 hr pulse-labelling). Partial hepatectomy was carried out [10] between 8–9 a.m. under light ether narcosis using groups of 6 Wistar female rats (175g). The labelled compounds were injected i.p. in a maximal vol 0.3 ml 2 hr before killing. The details are given in legends to the corresponding figures and tables.

Metabolic conversion of 5-*aza*-2′-*deoxycytidine*-^{3}H

Transformation of the drug was followed both *in vivo* and *in vitro* using Ehrlich ascitic tumor cells. During *in vivo* experiments the drug was injected i.p. to groups of 4 mice (100 μCi/0.2 μmole per animal) 2 hr before killing. Ascitic cells were removed and extracted with 5 vol of cold 0.4M $HClO_4$. The acid soluble fractions were neutralized with 2M KOH, cooled and clear supernatant fractions after centrifugation (5000 × ***g***, 5 min, 2°C) containing the newly formed radioactive metabolites were analyzed chromatographically using Whatman No. 3 paper as indicated in Figs. 1 and 2. The suspension of washed ascitic cells was used to follow transformations of 5-aza-2′-deoxycytidine-^{3}H *in vitro*. Incubation was carried out at 37°C in 25 mM Tris–HCl buffer (pH 7.5) containing 25 mM KCl and 5 mM $MgCl_2$ in the presence of 0.1 mM 5-aza-2′-deoxycytidine-^{3}H. The extraction of radioactive metabolites from the cellular pool and separation procedures were the same as described above. The radioactive spots on chromatograms were localized with a Frieseke–Hoepfner scanner and identified according to the position of standards.

Incorporation of 5-*aza*-2′-*deoxycytidine*-^{3}H

The incorporation of the analogue and of pyrimidine precursors into the fraction of nucleic acids was followed in various tissues of mice and rats. 5-Aza-2′-deoxycytidine-^{3}H, thymidine(methyl-^{3}H) or deoxycytidine-2-^{14}C were injected i.p. 2 hr before killing to groups of 5–6 animals. The tissues under investigation were quickly removed and homogenized in a cooled glass homogenizer with a tight-fitting Teflon pestle in 10 vol of cold 0.25 mM KCl. One millilitre portions of the homogenates were mixed with 1 ml of 0.4M $HClO_4$, the suspensions centrifuged (5000 × ***g***, 5 min, 2°C) and sediments extracted 3 times under cooling with 4 ml of 0.2M $HClO_4$ to remove the acid soluble low-molecular-weight components. Resulting sediments were extracted with 5 ml of alcohol–ether (3 : 1), dried and dissolved at 80–100°C in 1 ml of formic acid. The radioactivity of aliquots was measured using liquid scintillation system Isocap/300 (Nuclear Chicago Division).

RESULTS

Metabolic transformation of 5-*aza*-2′-*deoxycytidine*-^{3}H *in Ehrlich ascitic cells*

After the administration of 5-aza-2′-deoxycytidine-^{3}H to mice with Ehrlich ascitic carcinoma the drug is rapidly metabolized giving rise to new metabolites in the tumour cells. Analyzing total acid soluble cellular pool of the labelled cells by paper chromatography in different solvent systems at least nine newly formed compounds was detected. Figure 1 shows the separation of radioactive material on Whatman No. 3 paper using isobutyric acid–ammonium hydroxide-water as the solvent system.

Individual spots (Fig. 1A) were identified in the following way. Spot 1 is supposed to contain higher 5′-phosphates of the analogue and of 5-aza-2′-deoxyuridine. The corresponding zone was eluted and rechromatographed as is shown in Fig. 1(B). According to their chromatographic characteristics spot 2 contains 5-aza-2′-deoxyuridine 5′-monophosphate, spot 3, 5-azauracil, spot 4, 5-aza-2′-deoxyuridine (or product of its degradation), spot 5, 5-aza-2′-deoxycytidine 5′-monophosphate, spot 6 unknown derivative of deoxyribose and spot 7 unreacted 5-aza-2′-deoxycytidine. Spots 8, 9 and 10 contain the products of degradation of 5-aza-2′-deoxycytidine which are presently studied by Dr. A. Piskala of this Institute who supplied also some of the necessary standards.

The incubation of Ehrlich ascitic cells in suspension in the presence of 5-aza-2′-deoxycytidine-^{3}H resulted in the formation of the same set of compounds (Fig. 2A) as above. The addition of tetrahydrouridine, an inhibitor of cytidine deaminase [11], modifies the pattern of the newly formed compounds (Fig. 2B). While the level of products of 5-aza-2′-deoxycytidine degradation is unchanged (spots 8, 9 and 10) there is almost no radioactivity in the area corresponding to 5-azauracil (spot 3) and 5-aza-2′-deoxyuridine (spot 4). The radioactivity is localized mainly in the zone of 5-aza-2′-deoxycytidine 5′-monophosphate (spot 5) and of

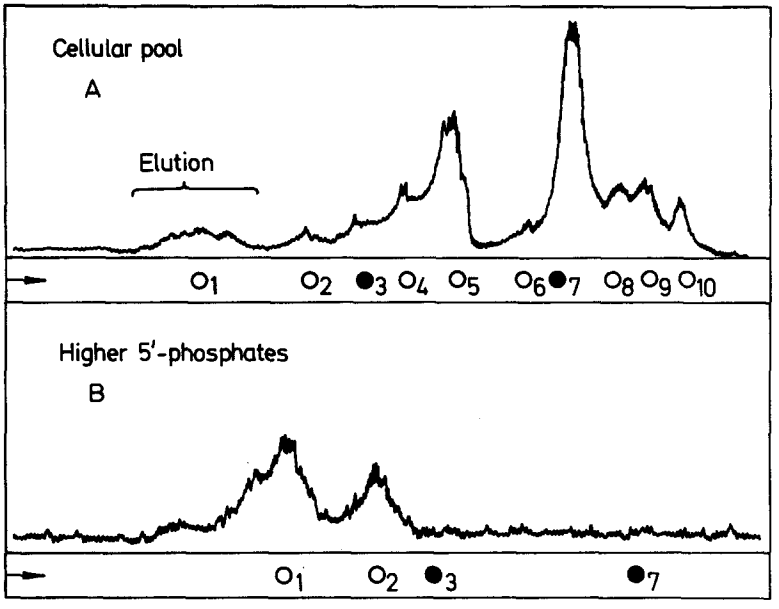

Fig. 1. *Radioactive metabolites in cellular pool of Ehrlich ascitic cells after* in vivo *administration of 5-aza-2'-deoxycytidine-*^{3}H.

Tritium labelled drug (200 μCi/0.2 μmole *per animal*) *was injected i.p. to* 4 *female mice* 9 *days after i.p. transplantation of* 1.10^7 *ascitic cells. Thirty minutes later the animals were killed, ascitic cells removed, extracted and the extract separated* 16 hr (*A*) *on Whatman No.* 3 *paper in a solvent system composed of isobutyric acid–ammonium hydroxide–water. The zone of higher* 5'*-phosphates was cut out, eluted with water and separated* 22 *hr on Whatman No.* 1 *paper using the same solvent system* (*B*).

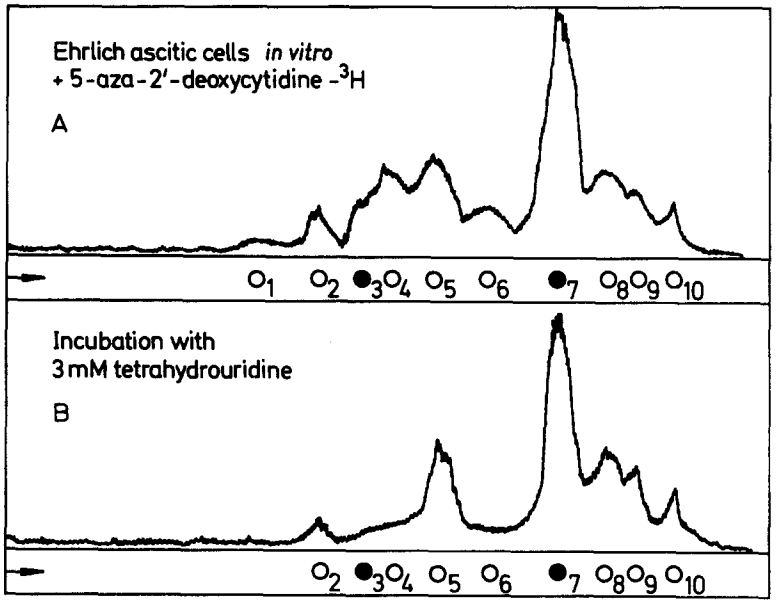

Fig. 2. *Effect of tetrahydrouridine on metabolic transformation of 5-aza-2'-deoxycytidine-*^{3}H *in suspension of Ehrlich ascitic cells.*

Ascitic cells collected from 5 *mice* 9 *days after transplantation were washed with* 0.9% *NaCl and suspended in* 25 mM *Tris*-HCl *buffer* (*pH* 7.5) *containing* 25 mM KCl *and* 5 mM $MgCl_2$. *The incubation was carried out at* 37°C *for* 20 *min in total volume of* 1 ml *in the presence of* 0.1 mM 5-*aza*-2'-*deoxycytidine-*^{3}H (200 μCi) *without* (*A*) *or with* (*B*) 3 mM *tetrahydrouridine. Extraction of the acid-soluble cellular pool and its chromatographic separation were carried out as in Fig.* 1.

5-aza-2′-deoxyuridine 5′-monophosphate (spot 2).

The time course of the uptake of the label into the fraction of nucleic acids during the incubation of Ehrlich ascitic cells in suspension with 5-aza-2′-deoxyxytidine-^{3}H is shown in Fig. 3. Somewhat lower uptake of the label observed in the presence of tetrahydrouridine apparently indicates that both 5-azacytosine and 5-azauracil nucleotides are taken up into cellular nucleic acids. The low stability of 5-azapyrimidines [12, 13] complicates the isolation and chemical characterization of nucleic acids with incorporated 5-azauracil or 5-azacytosine [14].

Preferential incorporation of 5-aza-2′-deoxycytidine-^{3}H into DNA in the spleen and the thymus of AKR mice with lymphatic leukemia

In earlier studies on the distribution [6] and inhibitory mechanism [7, 8] of 5-aza-2′-deoxycytidine in mice an increased uptake of the drug into lymphatic tissues was observed. In this study we followed the drug uptake and its distribution in tissues of AKR mice with lymphatic leukemia during the terminal stage of the disease.

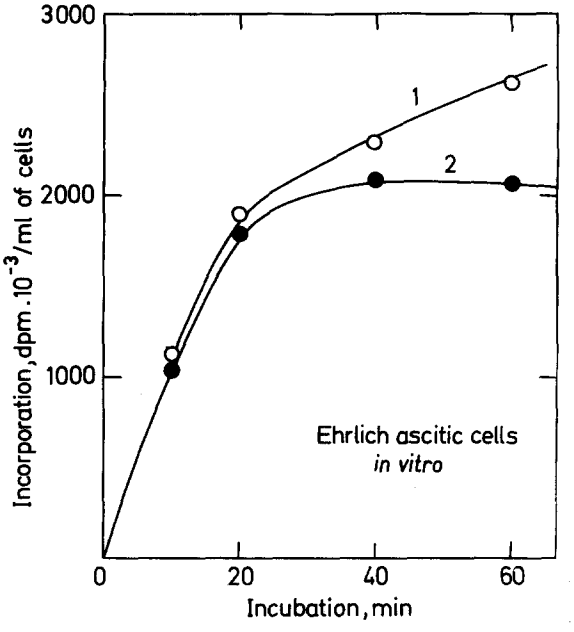

Fig. 3. In vitro *incorporation of 5-aza-2′-deoxycytidine-^{3}H into nucleic acids in Ehrlich ascitic cells.*

Ascitic cells from 10 animals (9 days after transplantation) free of erythrocytes and washed with cold 0.9% NaCl were suspended in 10 ml *of* 25 mM *Tris*–HCl buffer (*pH* 7.5) *containing* 25 mM KCl *and* 5 mM $MgCl_2$. *Incubation was carried out at* 37°C *in* 1 ml *portions after the addition of* 0.1 μmole *5-aza-2′-deoxycytidine-^{3}H* (200 μCi) *without* (1) *or with* (2) 3 *mM tetrahydrouridine. The incorporation of the label into the fraction of nucleic acids was measured as described in Material and Methods.*

From the data presented in Table 1 it is evident that thymidine-^{3}H is incorporated in control AKR mice about 11 times more effectively into nucleic acids in the spleen than in the liver. In leukemic animals the uptake of thymidine-^{3}H by the liver was 12 times (calculated per 1 g of the tissue) and 17 times (calculated per the liver) higher, respectively, than in the liver of intact animals. The labelling of nucleic acids, calculated per 1 g of the spleen and especially of the thymus of mice with lymphatic leukemia was lower than in the corresponding tissues of control AKR mice. However, the total uptake of thymidine-^{3}H into spleen nucleic acids was significantly increased (Table 1).

Using 5-aza-2′-deoxycytidine-^{3}H, the uptake of the radioactivity in the liver and kidney of intact AKR mice was about twice as high as that with thymidine-^{3}H. Nevertheless, the incorporation of the drug into nucleic acids in the spleen of intact animals was at least 25 times higher than in the liver (Table 2). While the uptake of thymidine-^{3}H was only about 4 times higher in the thymus than in the liver (Table 1) 5-aza-2′-deoxycytidine-^{3}H was taken up into thymus nucleic acids 12 times more effectively than in the liver (Table 2). In contrast to 12-17 fold enhancement of thymidine-^{3}H incorporation into leukemic liver the utilization of 5-aza-2′-deoxycytidine-^{3}H was enhanced 3–4 times only. Similarly as with thymidine-^{3}H the labelling of nucleic acids by the analogue in the spleen of mice with lymphatic leukemia was increased.

The ratio of the labelling of infiltrated livers, spleen and thymus with 5-aza-2′-deoxycytidine-^{3}H and thymidine-^{3}H, taking into account the labelling of the tissue and supposing the same radioactivity of administered compounds (see Tables 1 and 2) is 0.3–0.5, 4.2–5.5 and 8.0–11.5, respectively, indicating the preferential uptake of the analogue into lymphatic system.

To distinguish the uptake of 5-aza-2′-deoxycytidine into DNA and other molecules present in the acid-insoluble fraction the selective inhibitory effect of cytosine arabinoside on semiconservative replication of DNA [15, 16] was made use of. The data presented in Table 3 show that the incorporation of both thymidine and 5-aza-2′-deoxycytidine into the fraction of nucleic acids in the spleen and the thymus of leukemic mice is markedly depressed by the simultaneous administration of this drug. In the liver the inhibitory effect of cytosine arabinoside is much lower indicating the uptake of the label from both compounds into other molecules than only DNA. This is not surprising in view of the

Table 1. Incorporation of thymidine-^{3}H in tissues of AKR mice with lymphatic leukemia

	Incorporation (%)					
	dis/min 10^{-3}/g tissue ± S.E.			dis/min 10^{-3}/organ ± S.E.		
Tissue	Control AKR	Lymphatic leukemia		Control AKR	Lymphatic leukemia	
Liver	54.4 ± 6.1	640.0 ± 63.7	(1175)	54.7 ± 6.5	910.0 ± 85.5	(1672)
Kidney	63.2 ± 5.4	59.7 ± 4.6	(94.4)	17.0 ± 1.4	15.9 ± 1.2	(93.6)
Spleen	590.0 ± 56.0	500.5 ± 62.5	(84.8)	53.2 ± 6.1	114.6 ± 10.5	(216)
Thymus	215.0 ± 24.4	107.5 ± 21.0	(50.0)	17.2 ± 2.3	9.8 ± 2.2	(57.0)

Groups of 6 female AKR mice (24g) 7 days after s.c. transplantation with 2.10^7 leukemic cells were injected i.p. with thymidine (methyl-^{3}H) (50 μCi/0.2 μmole per animal) and sacrificed 2 hr after the injection. The incorporation of the label into the fraction of nucleic acids in individual tissues was measured as described in Material and Methods. Controls are taken as 100%.

Table 2. Incorporation of 5-aza-2'-deoxycytidine-^{3}H in tissues of AKR mice with lymphatic leukemia

	Incorporation (%)					
	dis/min 10^{-3}/g tissue ± S.E.			dis/min 10^{-3}/organ ± S.E.		
Tissue	Control AKR	Lymphatic leukemia		Control AKR	Lymphatic leukemia	
Liver	254 ± 35	753 ± 93	(296)	267 ± 31	1 037 ± 86	(389)
Kidney	298 ± 28	309 ± 33	(104)	75 ± 6	74 ± 7	(99)
Spleen	6 439 ± 385	6 120 ± 510	(95)	545 ± 44	1 420 ± 117	(261)
Thymus	3 088 ± 270	2 720 ± 304	(88)	248 ± 30	250 ± 21	(100)

Groups of 5 female AKR mice (26 g) 7 days after s.c. inoculation with 2.10^7 leukemic cells were injected i.p. 2 hr before killing with 5-aza-2'-deoxycytidine-^{3}H (125 μCi/0.2 μmole per animal). Controls are taken as 100%.

Table 3. Inhibition of thymidine-^{3}H and 5-aza-2'-deoxycitidine-^{3}H incorporation in various tissues of leukemic mice with cytosine arabinoside

	Incorporation, dis/min 10^{-3}/g tissue ± S.E. (%)		
Tissue	Control	Cytosine arabinoside	
	Thymidine-^{3}H		
Liver	771 ± 78	362 ± 65	(46.9)
Spleen	851 ± 68	51 ± 5	(6.0)
Thymus	161 ± 20	20 ± 3	(12.4)
	5-Aza-2'-deoxycytidine-^{3}H		
Liver	720 ± 108	423 ± 36	(58.8)
Spleen	9 350 ± 415	1 066 ± 53	(11.4)
Thymus	3 036 ± 216	712 ± 102	(25.8)

Groups of 5 male AKR mice (25 g) 7 days after inoculation with 2.10^7 leukemic cells were injected i.p. with cytosine arabinoside (3 μmole) simultaneously with thymidine (methyl-^{3}H) (50 μCi/0.2 μmole per animal) or 5-aza-2'-deoxycytidine-^{3}H (150 μCi/0.2 μmole per animal) 2 hr before killing. Controls are taken as 100%.

known metabolic conversion of 5-aza-2'-deoxycytidine into 5-azauracil [7] which is transformed to 5-azauridine 5'-monophosphate [17] and incorporated (5% of the incorporation of 5-aza-2'-deoxycytidine-^{3}H) into RNA. It is also known that tritium from thymidine-^{3}H can appear in macromolecular species other than DNA, especially in the liver [18].

Low incorporation of 5-aza-2'-deoxycytidine-^{3}H by proliferating hepatocytes in regenerating rat livers

The changes taking place after partial hepatectomy are characterized by an early increase of template activity of proliferating hepatocytes [19] followed 18–24 hr later by a pronounced enhancement of the synthesis of DNA and mitotic activity [20]. The data presented in Table

4 show that while thymidine and deoxycytidine are incorporated into liver DNA 24 hr after partial hepatectomy 9 and 12 times more effectively, the uptake of 5-aza-2′-deoxycytidine is not increased. However, in intact rat livers the incorporation of tritium labelled analogue was much higher than that of thymidine-^{3}H. This is probably due to the uptake of the label from 5-aza-2′-deoxycytidine-^{3}H into some other classes of cellular macromolecules than DNA. In the thymus of rats 24 hr after partial hepatectomy the utilization of thymidine, deoxycytidine and especially of 5-aza-2′-deoxycytidine was significantly decreased.

The treatment of rats by 5-azacytidine prior to partial hepatectomy results in the enhancement of thymidine incorporation into DNA in 24 hr regenerating livers [21]. Under similar conditions the utilization of the label from 5-aza-2′-deoxycytidine-^{3}H remained unchanged (Table 5). 5-Azacytidine pretreatment results in a further depression of thymidine-^{3}H and especially of 5-aza-2′-deoxycytidine-^{3}H incorporation into nucleic acids in the spleen and thymus.

DISCUSSION

5-Aza-2′-deoxycytidine was prepared synthetically [22] in 1964. While the corresponding ribonucleoside, 5-azacytidine, has been intensively studied since 1964 (e.g. [23, 24]) and is presently used with success in the treatment of various forms of human leukemias [25–27], 5-aza-2′-deoxycytidine has not yet found an application in the clinic. This is partly due to a limited supply of the deoxy analogue and mainly to a limited knowledge of the inhibitory mechanism of its action.

In mammalian tissues 5-aza-2′-deoxycytidine inhibits the phosphorylation of deoxycytidine in the acid-soluble cellular pool and depresses the incorporation of this pyrimidine into DNA [8]. At the same time the incorporation of the analogue into DNA molecules in the lymphatic system was observed. It is of interest that deoxycytidine affects the uptake of the analogue into nucleic acids to a lesser degree than 5-aza-2′-deoxycytidine the incorporation of deoxycytidine [7]. Following the administration of 5-aza-2′-deoxycytidine to mice the spleen of the

Table 4. Differences in incorporation of thymidine-^{3}H, deoxycytidine-2-^{14}C and 5-aza-2′-deoxycytidine-^{3}H in the liver of intact and partially hepatectomized rats

Administered	Incorporation, dis/min 10^{-3}/g tissue ± S.E. (%)		
	Intact	Partially hepatectomized	
Thymidine-^{3}H	31.1 ± 5.2	289.6 ± 28.8	(930)
Deoxycytidine-2-^{14}C	6.0 ± 0.7	73.2 ± 9.4	(1220)
5-Aza-2′-deoxycytidine-^{3}H	207.0 ± 22.5	218.2 ± 22.5	(105)

Groups of 6 intact or partially hepatectomized female rats (175–180g) 24 hr after operation were given 2 hr before killing thymidine(methyl-^{3}H) (100 μCi/0.2 μmole per animal), deoxycytidine-2-^{14}C (6 μCi/0.2 μmole per animal) or 5-aza-2′-deoxycytidine-^{3}H (100 μCi/0.2 μmole per animal). Intact animals are taken as 100%.

Table 5. Changes in the incorporation of thymidine-^{3}H and 5-aza-2′-deoxycytidine-^{3}H in 5-azacytidine-treated rats subjected to partial hepatectomy

Tissue	5-Azacytidine treatment	Incorporation, dis/min 10^{-3}/organ ± S.E. (%)			
		Thymidine-^{3}H		5-Aza-2′-deoxycytidine-^{3}H	
Regenerating liver	−	452.5 ± 43.8	(100)	342.7 ± 27.2	(100)
	+	678.3 ± 41.9	(150)	360.0 ± 16.8	(105)
Spleen	−	76.3 ± 12.0	(100)	354.0 ± 42.6	(100)
	+	41.8 ± 4.9	(54.8)	75.7 ± 10.4	(21.4)

Groups of 5 female rats (175–185g) were given i.p. at 24 hr intervals two doses of 5-azacytidine (6 μmole per 100g) or 0.9% NaCl and 24 hr later partial hepatectomy was carried out. Incorporation of thymidine(methyl-^{3}H) (100 μCi/0.2 μmole per animal) or 5-aza-2′-deoxycytidine-^{3}H (100 μCi/0.2 μmole per animal) into the fraction of nucleic acids was measured 24 hr after operation during a 2 hr pulse.

treated animals had much lower capacity to incorporate thymidine and displayed lower activity of thymidine and thymidylate kinases [7].

There are several findings indicating that the mode of action of 5-aza-2′-deoxycytidine is different from that of 5-azacytidine [7, 8, 23]. Recently the compounds originating from the tritium labelled drug were detected in the urine of mice already 4 hr after its administration. Beside several unidentified components 5-aza-uracil and guanidine were isolated from the urine in pure state and identified [7]. The transformation of 5-aza-2′-deoxycytidine in mice as proposed on the basis of compounds detected in the urine involves the deamination and splitting of deaminated deoxynucleoside resulting in the formation of 5-azauracil.

In the present study we used Ehrlich ascitic cells to follow the conversion of 5-aza-2′-deoxycytidine-^{3}H. From the data presented in Figs. 1 and 2 it is evident that the analogue is partly deaminated to 5-aza-2′-deoxyuridine and both fraudulent deoxynucleosides are phosphorylated not only to 5′-monophosphates but also to higher 5′-phosphates (Fig. 1B). Since tetrahydrouridine depresses the deamination of 5-aza-2′-deoxycytidine (Fig. 2B) and since 5-aza-2′-deoxyuridine 5′-monophosphate is present in the acid-soluble pool of the treated cells the deamination at the nucleotide level is probable. Using partially purified enzyme extract from calf thymus the formation of both anomalous 5′-monophosphates was observed also in an *in vitro* system (unpublished).

5-Aza-2′-deoxycytidine is incorporated into the fraction of nucleic acids. However, there are tissue differences in the uptake of tritium labelled analogue and of thymidine (Tables 1 and 2). While thymidine is incorporated into the liver of AKR mice with lymphatic leukemia to a larger extent than 5-aza-2′-deoxycytidine the latter compound is preferentially incorporated into spleen and thymus nucleic acids. Cytosine arabinoside markedly depressed the incorporation of both compounds in the spleen and the thymus indicating the incorporation of the label into DNA (Table 3). The lower inhibitory effect of cytosine arabinoside on the uptake of the label in infiltrated livers suggests the labelling of other molecules than only DNA.

Lower incorporation of 5-aza-2′-deoxycytidine-^{3}H than that of thymidine-^{3}H was also observed in proliferating hepatocytes in regenerating rat livers (Tables 4 and 5). Again, the higher uptake of the analogue (but less pronounced than in mice) was observed in the spleen and the thymus of intact and partially hepatectomized rats (Table 5).

These findings speak in favour of the increased affinity of 5-aza-2′-deoxycytidine for the lymphatic system and the potential usefulness of the drug against malignancies of the lymphatic system. Up to now 5-aza-2′-deoxyxytidine has not been used in the clinic in distinction to 5-azacytidine which is effective in acute myelogenous leukemia [1, 2, 27]. It remains to be seen whether in lymphatic leukemia which is less responsive to 5-azacytidine the deoxy analogue will be more efficient.

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Estradiol Binding by Human Breast Carcinoma Cytosols: The Influence of Commonly Used Reference Parameters on the Results

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Abstract—*The estradiol binding by human breast carcinoma cytosols was measured by saturation analysis and antiestrogen inhibition. The results were related to the weight of the tissue, the total protein content of the cytosol, the tissue protein content of the cytosol and the DNA content of the tissue. Taking estrogen binding/*mg *total protein estimated by saturation analysis as reference, the tumors were classified into estrogen receptor positive, estrogen receptor negative or borderline cases. Comparison with the estrogen binding related to the other tissue parameters or obtained by antiestrogen inhibition showed identical tumor classification in about* 85% *of cases.*

INTRODUCTION

THE ESTIMATION of estrogen receptors in the tumor has become an important factor in determining the treatment of disseminated breast carcinoma. Only patients with receptor-positive tumors have a significant chance of responding to endocrine therapy. It is, therefore, necessary to be aware of the numerous problems that are encountered when the receptor proteins are estimated [1–3]. One of the variables that has not received any attention so far is the influence of the various reference parameters commonly used to express the estradiol binding results. While comparisons between tissue slice and tissue cytosol results have been reported [4, 5] correlations between estradiol binding results obtained by saturation analysis and those obtained by antiestrogen inhibition have not been studied nor has the estradiol binding expressed per mg protein been compared with the binding per g tissue wet weight or per mg DNA. Do all these different ways of calculating the results agree with regard to classifying a carcinoma as receptor-positive or receptor-negative, or as a borderline case?

In this report estradiol binding by breast carcinoma cytosols was studied by both estradiol saturation and by antiestrogen inhibition, and the binding results were related to total cytosol protein, to the cytosol protein proper (i.e. not including the protein contamination from blood plasma), to DNA and to the wet weight of the tissue.

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MATERIAL AND METHODS

Preparation of cytosol

Homogenization of the tissue and preparation of the high-speed supernatant has been described before [6]. The cytosol was used for binding studies and for the estimation of protein, while the pellet was used for DNA determinations.

Estradiol binding by saturation analysis

Binding of estradiol to the cytosol receptor was studied over a concentration range of 50 pg to 5050 pg per ml cytosol using a constant amount of 50 pg tritiated estradiol and increasing amounts of unlabelled estradiol as described before [7]. Incubation was at 25°C for 1 hr followed by treatment with dextran-coated charcoal to remove excess steroid and loosely bound steroid. Receptor-bound estradiol-17β was determined as the difference between estradiol binding (in fmole/mg protein) at the lowest and at saturating steroid concentrations. From the Scatchard plot of the results the association constant of the estradiol-receptor

interaction and the number of binding sites were also calculated.

Estradiol binding in the presence of antiestrogen

Cytosol was incubated in the same way with 50 pg tritiated estradiol-17β in the absence and presence of 23,000 pg CN 55945 (Parke-Davis Co.)[8]. Receptor bound estradiol-17β was calculated as the difference between estradiol binding in the absence and presence of the antiestrogen.

Protein estimation

Total protein in the cytosol was measured according to Lowry *et al.* [9]. Plasma albumin in the cytosol was determined by immunodiffusion [10] using LC (low concentration) Partigen Albumin plates (Behringwerke A.G., Marburg, Germany). Tissue protein was then calculated as total protein minus 1.666 × plasma albumin.

DNA-estimation

The pellet and the fat layer obtained after high-speed centrifugation of the tissue homogenate were weighed. A half a gram of the pellet (or less) was redispersed by vigorous shaking in 5 ml of 5% trichloroacetic acid. The suspension was heated at 80%C for 20 min, then cooled and centrifuged for 5 min at 3800 rev/min, 0.1–1.0 ml aliquots of the supernatant were used for the color reaction and made up to 2 ml with 5% trichloroacetic acid. The pellet was re-extracted twice with fresh 5 ml portions of 5% trichloroacetic acid. Calf thymus DNA (Calbiochem) dissolved in 0.001 N NaOH was used as standard over a concentration range of 4–400 μg/4 ml. For the color reaction [11] equal parts of 0.08% indole in distilled water and 4 N HCl were mixed and 2 ml of this mixture were added to the DNA standards or pellet extracts. The solution was mixed on a vortex and heated in a boiling water bath for 15 min, during which time the tubes were shaken twice. The tubes were then cooled in ice water and extracted several times with 1 ml chloroform to remove the non-specific pink color, until the chloroform layer remained colorless. The optical density of the aqueous phase was measured at 490 μm using a 1 cm cuvette.

RESULTS

1. *Comparison of estradiol binding estimated by estradiol saturation analysis or by antiestrogen inhibition*

Estradiol binding by human breast carcinoma cytosol was estimated by saturation analysis, the difference between binding (in fmole/mg protein) at the lowest (50 pg estradiol/ml cytosol) and the highest (5050 pg estradiol/ml cytosol) concentrations being the amount of receptor-bound estradiol. Estradiol binding obtained in this way was used as reference for all comparisons and was also used to calculate association constant and number of binding sites. This is schematically shown on Fig. 1.

In the antiestrogen method, receptor-bound

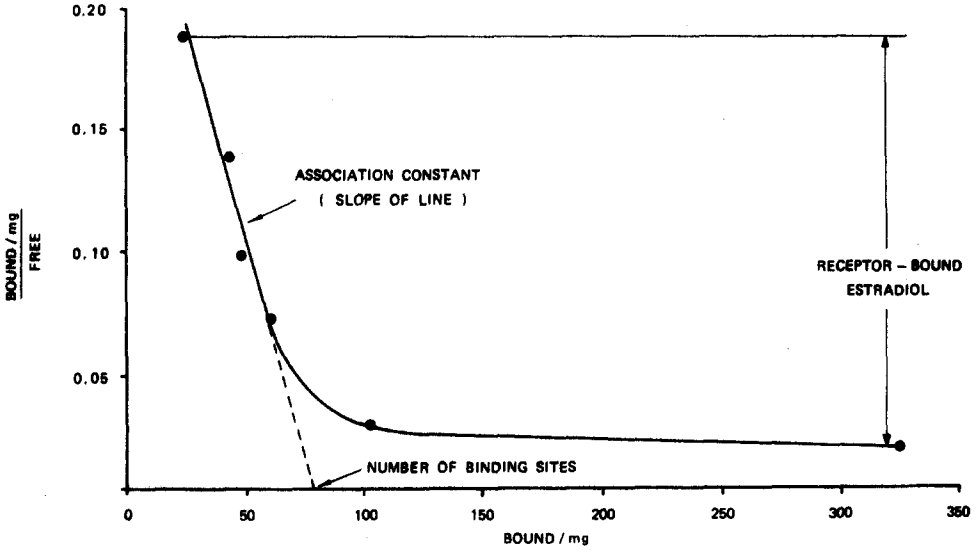

Fig. 1. Estimation of receptor bound estradiol, association constant and number of binding sites. The receptor-bound estradiol is calculated as the difference between the first and the last experimental points as shown schematically on the chart. The values used were not the derived figures of the ordinate (bound/mg)/free but the actual binding results in fmole estradiol bound/mg protein. The scale of the abscissa is in fmole.

estradiol was calculated as the difference between estradiol binding in the absence and in the presence of excess antiestrogen.

In Table 1, estradiol binding by human breast carcinoma cytosols, estimated by estradiol saturation (column A) and by antiestrogen inhibition (column B) is expressed per mg of total protein of the cytosol.

Breast carcinomas fall into 2 groups with regard to estradiol binding properties. One

Table 1. Estradiol binding related to different tissue parameters. ER+, ER±, ER− is the classification of the tumor based on estradiol binding in fmole/mg *total protein*

Tumor	Estradiol binding A	B	C	D	E
Receptor positive (ER +)					
FR-1	15.15	6.62	23.30	316.3	152.9
MB-1	13.84	5.97	19.94	325.5	120.4
MG-1	39.97	9.00	59.80	1138.3	446.0
MO-2	24.75	12.45	37.40	667.4	363.0
RA-1	56.20	−3.80	83.50	1551.1	—
RO-3	10.75	2.78	12.81	425.7	109.0
RU-1	31.49	19.45	—	644.9	—
SH-1	19.24	12.75	22.95	223.7	316.0
SM-1a	14.98	7.84	21.74	316.1	127.5
TA-1	45.54	26.20	81.05	1056.5	281.0
Receptor borderline (ER ±)					
BI-1	6.80	5.24	10.65	114.2	63.4
CO-1	6.49	3.83	9.61	135.5	—
DA-1	4.78	3.64	9.67	70.0	15.3
LE-2	7.51	4.87	10.09	183.4	37.1
MI-1	6.08	4.54	14.18	240.8	—
PO-1	6.60	4.10	—	155.2	—
Receptor negative (ER −)					
BA-1	0.24	1.27	0.39	5.1	13.4
BL-1	0.21	0.05	0.33	4.7	—
BO-1	−1.37	−0.42	−2.39	−24.1	—
BR-1	1.86	−3.90	3.50	37.5	25.7
CR-1	0.33	0.48	0.94	7.9	—
CR-2	1.24	2.59	7.20	15.9	79.4
DO-1	2.38	0.03	3.64	77.1	47.5
FL-1	2.82	2.71	10.50	37.2	11.4
GL-1	2.25	0.91	3.74	60.3	14.5
HA-2	−0.42	−2.02	−0.58	−6.8	—
KE-2	0.37	—	0.59	20.7	—
MA-2	1.98	1.94	4.09	75.9	45.0
MI-3	3.50	2.50	4.54	61.9	56.7
MO-1	0.91	0.60	2.43	38.2	13.4
MU-1	0.49	0.15	0.79	12.7	5.7
NE-1	0.85	−0.10	1.14	19.4	7.7
NI-1	1.80	0.62	3.16	36.3	—
NI-2	2.66	1.28	3.35	97.4	—
NI-3	2.06	0.46	3.05	48.7	18.1
NO-1	3.59	1.58	6.39	83.3	41.7
RE-1	2.65	—	5.04	78.4	—
RO-5	−0.38	−0.34	−0.58	−5.3	−22.0
SC-1	2.91	0.21	4.95	117.6	40.7
SE-1	−1.04	0.44	−1.76	−19.1	—
SM-1b	−0.28	0.05	−0.38	−11.7	−18.9
SQ-1	2.51	0.89	5.55	40.2	6.7
ST-3	1.78	0.01	3.37	28.4	12.4
ST-4	0.64	0.47	0.84	16.4	5.0
WE-1	0.25	1.47	—	5.0	1.3
WH-1	3.85	2.20	8.87	112.4	56.8
WH-2	3.90	1.37	6.92	138.8	133.8
WI-1	2.23	0.12	3.80	76.7	64.0

Column A is fmole/mg total protein, by estradiol saturation. Column B is fmole/mg total protein, by antiestrogen inhibition. Column C is fmole/mg tissue protein. Column D is fmole/g tissue (wet weight). Column E is fmole/mg DNA of tissue.

category of carcinomas behaves like a typical target organ (e.g. uterus), binds considerable amounts of estradiol and the binding is achieved by a limited number of receptor sites. The other group binds little estradiol, the binding is not saturable and is in all other aspects like the non-specific binding by a non-target tissue (e.g. skeletal muscle) [4]. The actual binding values assigned to ER+, ER± and ER− depend on a number of variables [2] and are somewhat arbitrary. In spite of this various groups working independently have come to very similar figures and have concluded that this classification is helpful in predicting the results of endocrine therapy for metastatic breast cancer [3].

If one defines binding values (obtained by saturation analysis) greater than 8 fmole/mg as estrogen receptor positive (ER+), values between 4–8 fmole/mg as borderline cases (ER±) and values below 4 fmole/mg total protein as receptor negative (ER−), the following tumors in Table 1 would be estrogen-dependent: FR-1, MB-1, MG-1, MO-2, RA-1, RO-3, RU-1, SH-1, SM-1a, TA-1. In this group the mean estradiol binding was 27.19 with a standard deviation of ± 15.53 and a range from 10.75 to 56.20 fmole/mg. The group of ER± cases consists of: BI-1, CO-1, DA-1, LE-2, MI-1, PO-1, with estradiol binding ranging from 4.78 to 6.80 and a mean and standard deviation of 6.38± 0.91 fmole/mg. All the remaining breast carcinomas were ER−. The mean estradiol binding in this group of 30 carcinomas was 1.48 fmole/mg with a standard deviation of± 1.45 and a range from −0.42 to 3.85. Using this grouping of the individual tumors and then applying it to the results obtained with the same tumors by antiestrogen inhibition shows general agreement (39/46) but also a number of discrepancies. All the tumors found to be ER− by estradiol saturation analysis would also be ER− by the antiestrogen inhibition method. The mean binding and standard deviation in this group was 0.58± 1.33 fmole/mg with a range from −3.90 to 2.71. The mean binding was significantly lower than that obtained by estradiol saturation (t= 2.489, confidence limit> 98%). The same trend for lower results with the antiestrogen inhibition was observed in the ER± and the ER+ groups. Estradiol binding in the ER± group estimated by antiestrogen inhibition was 4.37± 0.62 fmole/mg (mean± standard deviation) which is significantly lower than the results for this group obtained by estradiol saturation (t= 4.451, confidence limit> 99.73%). The ER+ group bound 9.93± 8.44 fmole/mg (mean± standard deviation) estimated by antiestrogen inhibition, much less than obtained by estradiol saturation (t= 3.088, confidence limit> 99%).

Two results CO-1, DA-1 that were ER± by estradiol saturation, showed binding of less than 4 fmole/mg protein by antiestrogen inhibition and would have been classified ER−. In 5 more carcinomas estrogen receptors would have been underestimated (in relation to the classification obtained by estradiol saturation analysis). Three showed intermediary levels (FR-1, MB-1, SM-1a) which would have put them into the ER± group and in two (RA-1, RO-3) antiestrogen inhibition would have classed the tumor as ER−, while estradiol saturation analysis grouped them as ER+. These results are summarized in Table 2.

Table 2. Comparison of estradiol binding of 46 breast carcinoma cytosols estimated by estradiol saturation (reference) and by antiestrogen inhibition

	ER+ n=10	ER± n=6	ER− n=30	Total n=46
Agreement	5	4	30	39
Underestimation	5	2	0	7
Overestimation	0	0	0	0

Underestimation means that binding based on antiestrogen inhibition would have placed the tumor in a group with less ER content; overestimation indicates that binding based on antiestrogen inhibition would have placed the tumor into a group with higher ER content. "n" is the number of cases.

The correlation coefficient for the pairs of results in columns A and B, Table 1 was 0.624 (confidence limit>99.9%) and the regression, y=1.004+0.271 x.

2. *Comparison of results of estradiol binding (estimated by estradiol saturation) expressed per* mg *total protein, per* mg *tissue protein, and per* g *tissue wet weight*

In addition to using 2 different methods of estimating receptor binding (estradiol saturation and antiestrogen inhibition) the values thus obtained have also been related to different reference parameters. In the earlier stages of receptor research the wet weight of the tissue was most commonly used for this purpose, later also the total protein content of the cytosol, and more recently the tissue protein and the DNA content of the tissue. In Table 1 the results of estradiol binding calculated per mg total protein (column A) are compared with the same results expressed per mg tissue protein (column C) and per g tissue wet weight (column D).

Since the results of columns A and D (Table 1) have different units these figures cannot be compared directly as those of columns A and B. Rather, the binding results per g tissue wet weight were grouped into ER+, ER± and

ER − based on the classification derived from the estradiol binding per mg protein. When this was done, a mean estradiol binding per g tissue of 40.22 fmole with a standard deviation of 42.02 and a range from −24.11 to 138.84 was found for the ER− carcinomas. This is significantly different from the group of ER± tumors (confidence limit >99.9%) which showed a mean binding of 149.87± 58.79/g tissue. The lowest result in this group (DA-1), however, would fit better into the ER− class, and the highest (MI-1) could also be in the ER+ tumors. Three individual results from the ER− class (WH-2, WH-1, SC-1) could also belong to the ER± sample. The mean binding in the ER+ group was 666.55 ± 443.92 fmole/g tissue. While this is significantly different from the ER± class (confidence limit >98%), one individual result (SH-1) would also fit into the ER± class. These findings are summarized in Table 3. The correlation coefficient for the comparison of binding per g tissue with binding per mg total protein was 0.980 (confidence limit >99.9%) and the regression $y = -3.368 + 25.25$ x.

Table 3. Comparison of estradiol binding of 48 breast carcinoma cytosols calculated per mg *total protein (reference) and per* g *wet weight of tissue*

	ER+ $n=10$	ER± $n=6$	ER− $n=32$	Total $n=48$
Agreement	9	4	29	42
Underestimation	1	1	0	2
Overestimation	0	1	3	4

Again, the designation of the tumors as ER+, ER± and ER− would have been identical in most instances (42/48) no matter if the estradiol binding was related to the weight of the tumor or to the total protein content of the tissue cytosol. In the few cases where the two methods differed, no trend towards over or underestimation was evident.

Part of the total protein concentration in the tissue cytosol is not due to tissue protein but represents a contamination by plasma proteins. The proportions will vary according to the vascularity of the tissue involved and, therefore, relating estradiol binding to total cytosol protein may result in an erroneous classification of the tumor. By estimating serum albumin in the cytosol and assuming 60% albumin in serum, the contribution of blood protein to the total cytosol protein can be assessed [12]. In the present series of tissue proteins in the cytosol made up an average of 59.5% of the total protein with a standard deviation of ± 14.1%. There was no statistically significant difference between the tissue protein in the ER+, ER± or ER− cytosols, nor was there any difference in the total proteins. In other words, the classification of the tumors into one of the 3 groups was not influenced by relating the estradiol binding to either total protein or tissue protein. Since the tissue protein is always less than the total protein, estradiol binding expressed per mg tissue protein (Column C, Table 1) will always be higher than the estradiol binding per mg total protein (Column A, Table 1). Estradiol binding per mg tissue protein in the ER+ group was 40.28 ± 27.43 fmole/mg tissue protein (mean ± standard deviation) and was higher than in the reference group but the difference was not statistically significant ($t = 1.273$, confidence limit 95%, 2.12). Similarly, the results related to tissue protein were higher than those related to total protein for both the ER± and ER− groups (10.84 ± 1.91 fmole/mg tissue protein, and 3.01 ± 3.06 fmole/mg tissue protein, respectively, $t = 4.657$, confidence limit >99% and $t = 2.485$, confidence limit >95%, respectively). These results are summarized in Table 4.

Table 4. Comparison of estradiol binding of 45 breast carcinoma cytosols calculated per mg *total protein (reference) and per* mg *tissue protein*

	ER+ $n=9$	ER± $n=5$	ER− $n=31$	Total $n=45$
Agreement	9	0	21	30
Underestimation	0	0	0	0
Overestimation	0	5	10	15

The correlation between estradiol binding per mg tissue protein and estradiol binding per mg total protein was highly significant (correlation coefficient 0.991, regression $y = 0.462 + 1.536$ x).

Once more, in the majority of cases (30/45) the classification would have been identical, whether the estradiol binding was related to tissue protein or total protein. In one third of the cases, however, the use of the tissue protein values as a reference for the estradiol binding results would have put the tumors into a group with a higher receptor content.

3. *Comparison of estradiol binding per* mg *DNA and per* mg *total protein*

The pellet obtained after high-speed centrifugation of the tissue homogenate was assayed for DNA, and the estradiol-binding results estimated in the supernatant were related to the DNA values by calculating the amount of DNA

per g tissue and then expressing the amount of estradiol bound per mg DNA. The results are given in Table 1, column E.

If the tumors are classified as ER ± or ER − on the basis of the estradiol binding results per mg protein (column A), the estradiol binding per mg DNA (column E) in the ER − group is 28.70 ± 34.59 fmole/mg DNA and that of the ER + group 239.48 ± 129.13 fmole/mg DNA. The small ER ± group is not statistically different from the ER − group (38.60 ± 24.09 fmole/mg DNA). In most cases the classification of the tumors would have been identical no matter whether estradiol binding was calculated per mg DNA or per mg protein. But close inspection of the results shows that some individual tumors would not be in the same group. Tumor WH-2 clearly belongs to the ER + group according to the estradiol binding per mg DNA, but is in the ER − group by its estradiol binding per mg protein. Tumors WI-1 and CR-2 that are ER − based on the reference method, would have been classified as ER ± by its estradiol binding per mg DNA; and tumors LE-2 and DA-1, both ER ± by the reference method would have been ER − when mg DNA was used as the basis for calculating the results. These differences and agreements are summarized in Table 5.

Table 5. *Comparison of estradiol binding of* 34 *breast carcinomas calculated per* mg *protein (reference) or per* mg *DNA*

	ER + $n=8$	ER ± $n=3$	ER − $n=23$	Total $n=34$
Agreement	8	1	20	29
Underestimation	0	2	0	2
Overestimation	0	0	3	3

The correlation between the results expressed per mg DNA and those expressed per mg total protein was highly significant (correlation coefficient 0.899, confidence limit > 99.9%, regression $y = 12.442 + 9.264\,x$.

4. *Comparison of tumor classification based on estradiol binding per* mg *protein or association constant of estradiol-receptor interaction*

It was suggested in an earlier paper [7] that the characterization of the estradiol-receptor interaction by saturation assay and Scatchard plot provides the best discrimination between hormone-dependent and hormone-independent breast carcinomas. This was based on evidence that the association constant—unlike estradiol binding per mg protein—was not influenced by partial saturation of the receptors due to endogenous estradiol, nor by partial loss of receptors due to freezing or storage. The Scatchard plot also provides a visual aid which makes the distinction between ER + and ER − tumors easier, since ER − tissues give a straight line with a very small slope or, depending on the scale, a straight line apparently parallel to the abscissa and ER + tissues a nonlinear curve (Fig. 1). Since detailed results of a comparison between association constant and binding per mg protein, based on a much smaller sample, were presented before [7], the individual results of the 177 tumors (which include the earlier results, results of the patients listed in Table 1 and additional tumors) have not been tabled, but are summarized only (Table 6).

Table 6. *Comparison of tumor classification in* 177 *patients, based on estradiol binding per* mg *protein (reference) and association constant of estradiol-receptor interaction*

	ER + $n=82$	ER − $n=95$	Total $n=177$
Agreement	78	89	167
Underestimation	4	0	4
Overestimation	0	6	6

As before, estradiol binding below 4 fmole/mg protein indicates ER −, between 4 and 8 fmole/mg ER ± and above 8 fmole/mg ER +. Based on the Scatchard plot a tumor is ER + if there is evidence for saturable binding sites (nonlinear curve) and ER − if saturation cannot be demonstrated (line parallel to abscissa) [7]. In most cases both methods result in an identical classification of the tumor and over and underestimation occur with about the same frequency. Since a borderline classification has not been defined for the association constants, 25 ER ± results from the estradiol binding per mg protein have not been included in Table 6. Of these results, 6 would have been receptor negative and 19 receptor positive by the Scatchard plot method.

DISCUSSION

It is reassuring to know that the distinction between estrogen receptor positive and estrogen receptor negative human breast carcinomas based on the binding of estradiol is identical in the large majority of cases (about 85%) even when the tissue parameters to which the results are related, are as diverse as tissue wet weight, total or tissue protein content of the cytosol,

DNA content of the tissue, and even though different procedures like antiestrogen inhibition or estradiol saturation are used to obtain the results.

In relatively few cases the results differed sufficiently from one another to change the tumor classification in comparison to the estradiol binding per mg total protein, which was arbitrarily used as a reference method. It is not known at present which results most accurately describe the receptor status of the tumor and most reliably predict the patient's response to endocrine therapy in those cases in which the same estradiol binding by one particular tumor, related to different tissue properties (weight, protein, DNA etc.), put the tumor into different categories (ER+, ER± and ER−). The results of the present study indicate that at least in some comparisons the trends are obvious. Estradiol binding given per mg tissue protein would "overestimate" receptors in comparison with results expressed per mg total protein (Table 4) and antiestrogen inhibition tends to comparatively "underestimate" receptors (Table 2). To obtain comparable results the binding limits for the groups (> 8 fmole/mg total protein for ER+, 4–8 for ER±, < 4 for ER− in the reference method) could be redefined for the two other methods just mentioned. In the remaining comparisons (Tables 3, 5 and 6) where results led to both higher and lower classifications compared with the standard method such adjustments cannot be made and the reasons for the differences are obscure.

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Osteosclerotic Myeloma with Polyneuropathy and Ectopic Secretion of Calcitonin

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Abstract—*The authors report the clinical observation of a patient with radiological features of an IgA with light chains type lambda osteosclerotic myeloma, associated with progressive sensorimotor polyneuropathy. Attention is drawn to these rare sclerotic forms of myeloma and the high frequency of associated neuropathies.*

In this case, marked hypercalcitoninemia was discovered with no other abnormality of the phosphocalcic balance. The authors advance the hypothesis of an ectopic secretion of calcitonin by tumoral plasma cells and discuss the potential role of this hormone in the pathogenesis of myeloma osteosclerosis.

In addition, the fixation of the M-Component on the peripheral nerve trunks is demonstrated by means of immunofluorescence techniques, thus directly supporting the immunological conception of dysglobulinemic neuropathies.

INTRODUCTION

MALIGNANT plasmocyte proliferations most often induce osteolytic and/or osteoporotic radiological lesions and, far less frequently, sclerotic bone lesions [1, 2].

The secretion of an osteoclast inhibiting factor or of an osteoblast stimulating factor has been advanced to explain this osteocondensation [3].

Chronic sensorimotor polyneuropathies are frequently associated with these rare sclerotic myelomas [4]. The pathogenesis of these dysglobulinemic polyneuropathies is not clearly known. Recent observations suggest that nerve alterations may be due to immunological mechanisms [5, 6].

MATERIAL AND METHODS

Case report

Mr. Leonard C . . . was born in 1915. He suffered from chronic phlebopathy of the lower limbs and labile hypertension. Since 1967, he has been complaining of paroxysmal positional vertigo, transient visual disorders, and of intermittent weakness, either of the left leg, or on the right side of the body.

An X-ray picture of the cervical spine showed metastatic-like homogeneous osteocondensations, located in the vertebral body of C3, in the left pedicle and in the posterior apophysis of C7 (Fig. 1a and b). An additional osteosclerotic lesion was discovered in the left postero-lateral portion of the vertebral body of D9.

On examination, a few hard, mobile, painless adenopathies were felt in the right supraclavicular fossa. Cardiopulmonary condition was in the normal range. There was no hepatomegaly nor splenomegaly.

Neurological examination

Moderate weakness was noticed in the distal muscle groups of the lower extremities. The muscle strength reflexes were normal in the upper limbs but the rotular reflexes were sluggish and the Achilleus reflexes were absent. There was no Babinski sign. Sensory examination revealed decreased vibratory sense in the distal portions of both the upper and the lower extremities. There was sock-like hypo-algesia in the lower limbs.

RESULTS

General investigations

The blood cells count was normal. Erythrocyte sedimentation rate was 20 mm/1st hr. Fibrinogen was 4.5 g/l and blood proteins were 67 g/100 ml. Electrophoresis showed 60.4%of albumin, 2.8% of alpha 1-globulins, 19.2% of gamma-globulins. Serum electrolytes and a fasting blood sugar level were normal. Renal and hepatic tests were normal. Serological tests for syphilis were negative. Serum alkaline

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and acid phosphates, hydroxyprolinuria were in the normal range. Glucose tolerance test was normal.

Phosphocalcic values were in the normal range except for calcitonin (CT) which was highly increased to 3000 pg/ml. (In normal subjects, the basal levels of CT are between 0 and 600 pg/ml of serum with a mean of 180 pg ± 150 [7].)

Biopsy of the enlarged lymph nodes showed pseudonodular massive plasmocyte infiltration of the medullary cords, with peripheral compression of the lymphatic follicles (Fig. 2).

These histological findings suggested the diagnosis of an osteosclerotic form of Kahler's disease, which was confirmed by the following techniques.

Serum IgA was twice higher than the normal value: 800 mg%. The IgG and IgM were normal. Immunoelectrophoresis showed a pathological monoclonal immunoglobulin (M-Component), identified as an IgA with light chains type lambda. Urine examination for proteinuria was negative. Conventional tests for Bence–Jones proteins in concentrated urine (× 100) were negative.

Light microscope examination of the bone marrow showed a medullary plasmocytosis at 7%, with many abnormal cells. Bone biopsy of C7 posterior apophysis confirmed the lesion and showed marked thickening of the spongy bone structures, with reduced medullary spaces containing a large population of plasmocytes and plasmoblasts, often bi-nucleate as already seen in the lymph nodes (Fig. 3).

Neurological investigations

The cerebrospinal fluid (CSF) was normal, except for a total protein count of 55 mg/100 ml. Electrophoresis of CSF proteins was normal. Immunoelectrophoresis was not obtained.

Electrodiagnostic studies showed marked alterations of motor nerve conduction velocity with no motor responses in both anterior tibial nerve trunks. In most distal muscles, electromyography showed fibrillation potentials at rest and decreased interference pattern on voluntary contraction.

Biopsies of cutaneous branches of the sural nerve, were examined by light-, electron- and immunofluorescent microscopy. No morphological modification was observed by light microscopy. On transverse semi-thin sections, the nerves were almost normal (Fig. 4). However, moderate demyelinisation was already evident on some trunks (Fig. 5). Focalized alterations of myelin sheaths were confirmed by electron-microscopic examination (Fig. 6). The morphological modifications were slight in the nerve and very partial in each nerve trunk. The amyelinic nerves seemed normal and the Schwann cells were not modified. The conjunctive structure of the nerve was morphologically intact and no fibrillar material suggesting amyloid could be found. There was no inflammatory infiltrate. The wall of some small arteries however, was thickened by an "onion bulb" proliferation of basal membranes which included cytoplasmic ramifications of endothelial cells (Fig. 7).

Fresh longitudinally cut fragments of the same nerve trunk were studied with the usual two-layer immunofluorescent technique. Fluorescein-conjugated anti IgG and anti IgM antiserums stained the preparations in a non-specific way and only a diffuse basal fluorescence was observed. In contrast, application of the fluorescein-conjugated anti IgA antiserum revealed intense staining of the perineurium and of the central portion of the nerve (Fig. 8). No specific labelling was obtained with any of the antiserums when tested on fragments of peripheral nerve trunks of three controls with osteolytic myelomas (two IgG myelomas, one IgA myeloma), but without clinical or electrical signs of polyneuropathy.

Therapy and follow-up

The patient was treated with a daily dose of cyclophosphamide (100 mg per os) and high doses of vitamin B complex. This therapy was continued from May 1974 to June 1976 with gradual and marked improvement of the sensori-motor polyneuropathy. In May 1975, after one year of therapy, the motor nerve conduction velocities became subnormal and, a motor response was again elicited in the anterior tibial nerves. The radiological control showed no variations in size and aspect of the previous lesions, nor the development of any other ones. Although the patient experienced a clear improvement in his clinical state, chemotherapy did not appear to modify the serum levels either of CT or of immunoglobulins [Table 1] while repeated immunoelectrophoresis of serum proteins showed the abnormal IgA spike throughout the clinical course.

DISCUSSION

This unusual observation raises many questions but we shall discuss the two major ones only: the osteosclerotic myeloma and the myelomatous polyneuropathies.

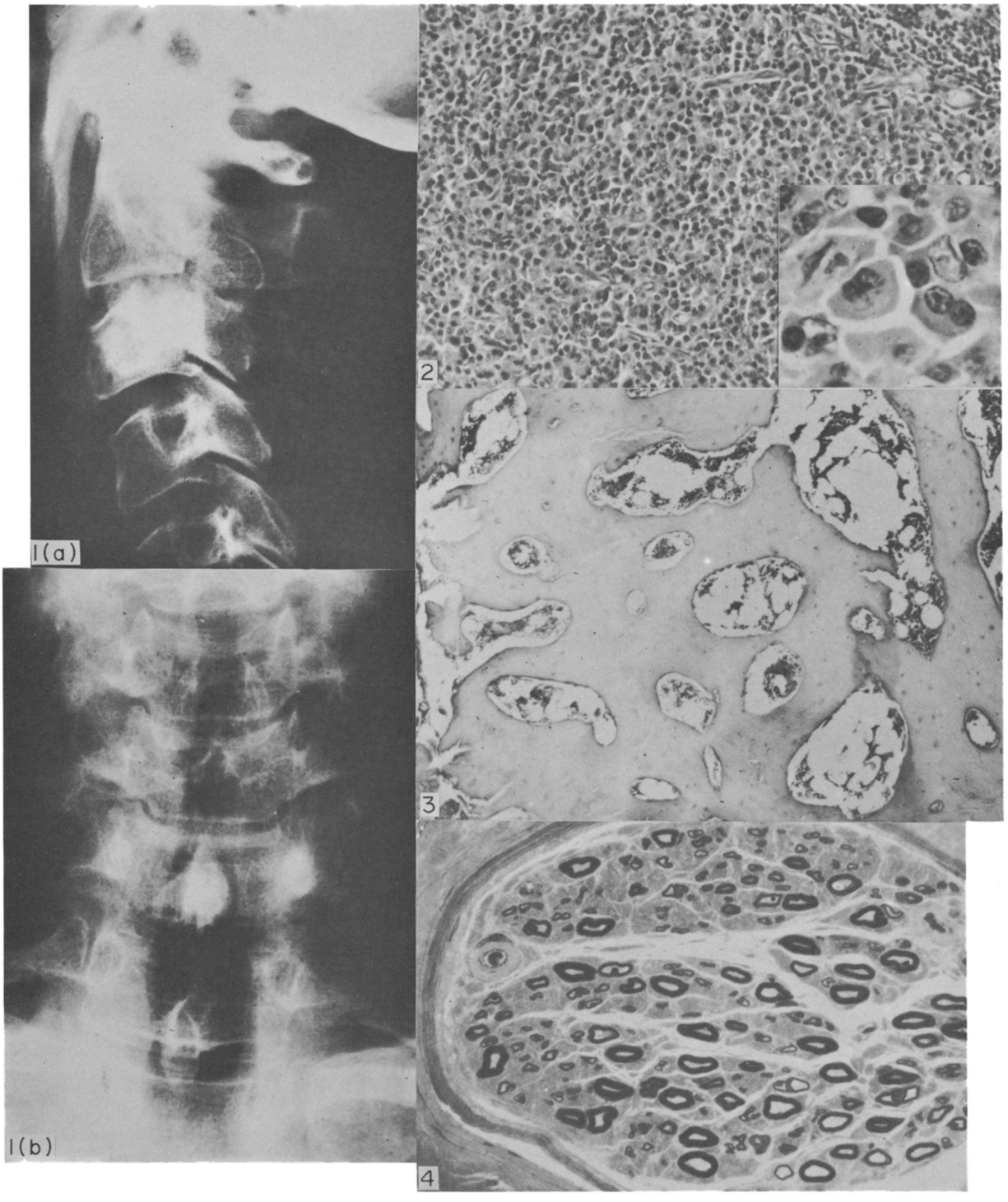


Fig. 1. *Radiograph of cervical spine revealing homogeneous osteocondensations at the level of: (a) C3 vertebral body, (b) C7 posterior apophysis and left pedicle.*

Fig. 2. *Microscopic section of lymph node showing striking infiltration by plasma cells at varying stages of maturity. Inset: detail of large and binucleated plasma cells* (H.E. 100 *and* 400 ×).

Fig. 3. *Microscopic section from C7 osteosclerotic posterior apophysis demonstrating thickened bony trabeculae and reduced marrow spaces* (H.E. 60 ×).

Fig. 4. *Transverse section of nerve demonstrating a normal population of large, medium and small myelinated and unmyelinated axons.* (*Toluidine blue:* 480 × .)

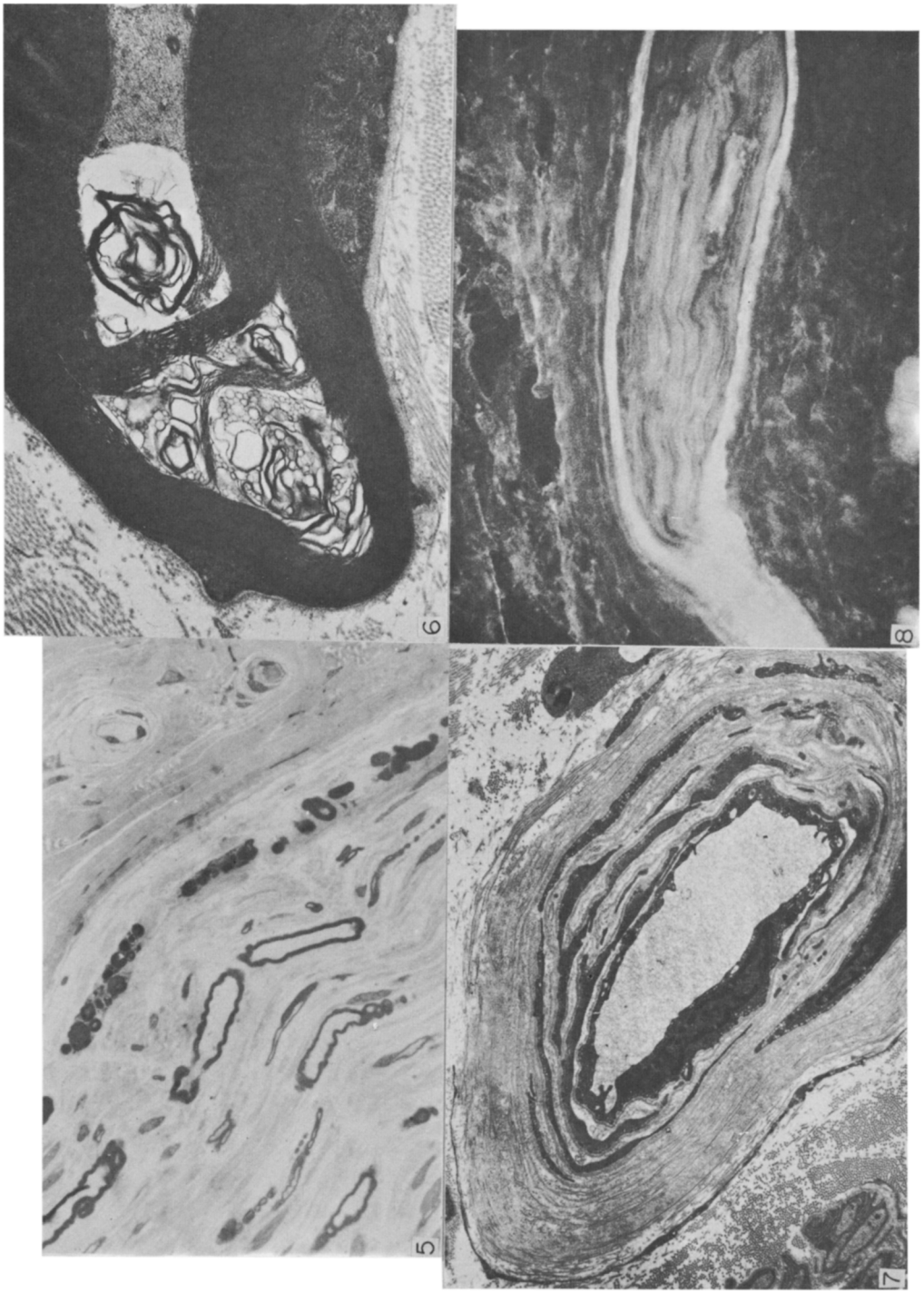


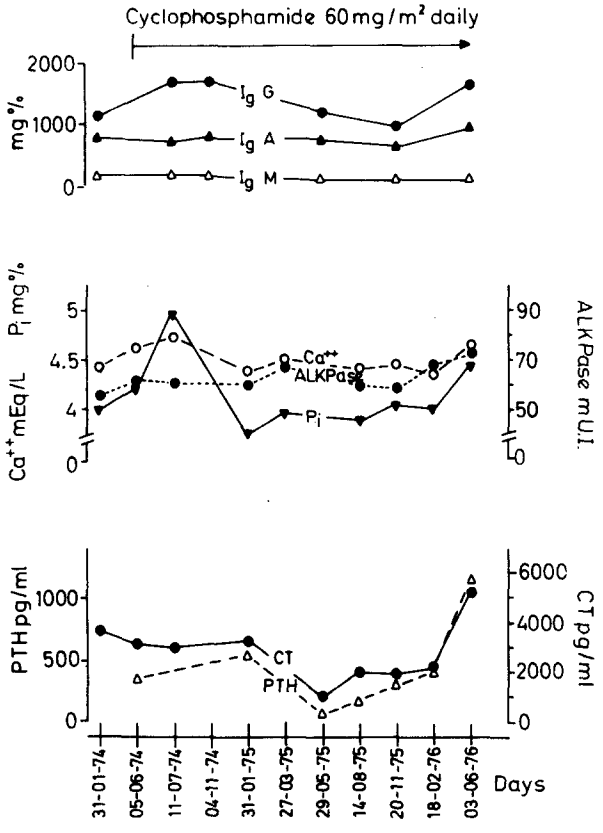
Fig. 5. ***Longitudinal section of a nerve segment in which partial demyelination is evident. (Toluidine blue:*** 600 ×).

Fig. 6. ***Partial fragmentation and vacuolisation of a large myelin sheath while the axon is still normal*** (10,000 ×).

Fig. 7. *Transverse section of a small artery in the nerve. Numerous basal membranes are intermingled with ramifications of endothelial cell cytoplasm* (7500 ×).

Fig. 8. *Longitudinal section of nerve fragment revealing strong immunofluorescence on perineurium and along surface of myelin sheaths when preparation is incubated with fluoresecein conjugated anti TgA anti serum* (160 ×).

Table 1. *Biological examination during a follow-up of two and a half years and beginning of an uninterrupted oral chemotherapy in May* 1974



(a) Upper graph: serum immunoglobulins; (b) Middle graph: serum calcium ○ – – ○, inorganic phosphorus ▼——▼, alkaline phosphatases ●---●: (c) Lower graph: parathormone △---△ and calcitonin ●——●.

I. Osteosclerotic myeloma

(A) *Radiological and clinical aspects.* These osteosclerotic forms of myeloma are very infrequent. Only 50 cases have been reported in the literature (for a general review, see [8–11]). Their radiological features have been reviewed in 37 observations by Bismuth *et al.* [3]. Four main lesions are noted. The most frequent are pictures of diffuse osteosclerosis (37%) and of focal osteocondensation which give a "mottled" aspect of the bone (30%). Less often, there are bony spiculations on the surface of the bone (20%) and lytic images surrounded by a ring of osteosclerosis (13%). Occasionally, these various lesions may be observed in the same patient and be associated with lytic lesions. Osteosclerotic lesions mainly involve the axial skeleton and unlike osteolytic lesions, tend to spare the skull.

The pathology of osteosclerosis is linked to thickening of spongious bone structures surrounding the foci of plasmocyte tumoral proliferation [3, 4, 8, 12–14].

(B) *Pathogenesis of the bone sclerosis.* The mechanism of bone sclerosis in myeloma is not clear. It has been suggested that the osteocondensation might be induced either by inhibition of osteoclastic resorption, or by osteoblastic hyperactivity in the tumoral foci [15] and might occur indirectly through the action of a hormonal or pseudo-hormonal (?) substance secreted by tumoral cells [3]. The very high levels of CT found in this case suggest that CT might be this hormonal substance.

Although, to our knowledge, such a hypercalcitoninemia has not yet been described in osteosclerotic myelomas, it is tempting to advance that CT could be the inductive factor of osteosclerosis in some cases of myelomas. In fact, some authors have demonstrated that CT inhibits bone resorption by reducing osteolysis [16, 17] and that CT is produced in large amounts by tumoral cells in thyroid medullary carcinomas, a condition in which bone metastases are frequently osteosclerotic [18]. An ectopic secretion by tumoral cells, a phenomenon already described in other neoplastic affections [19, 20] seems to be the most likely hypothesis to account for high levels of CT. To support this assumption, we are now measuring levels of CT in extracts of various tissues, particularly of lymph nodes invaded by tumoral plasmocytes (Fig. 2). Our preliminary results which will be published elsewhere, seem to support this mechanism.

Thus, this production of an "osteogenic" factor by the tumoral plasmocyte could be the very reverse of the mechanism involved in the bone resorption of osteolytic myelomas, where secretion of an osteoclast stimulating factor has been proved by Mundy *et al.* [21].

II. *Myelomatous polyneuropathies*

(A) *Frequency.* The incidence of chronic polyneuropathy varies in relation to the radiological picture of the myeloma. In the classical osteolytic or osteoporotic forms, polyneuropathies are observed in 2.7% [22] to 3.2% [23] of the cases. But, in a survey of 36 osteosclerotic myelomas, Mangalik *et al.* [9] found these complications in 40% of the cases. The frequency of polyneuropathy is evaluated at 20% in the course of single plasmocytomas [24].

No common factor has been noted to account for this increased incidence of neuropathy in sclerotic forms of myeloma. Compression of numerous nerve roots by sclerotic bone is doubtful because deformation of involved bones has not been observed, even in generalized sclerotic forms [3].

(B) *Clinical aspects.* According to Davis *et al.*

[24], manifestations of sensorimotor polyneuropathies can precede the diagnosis of myelomatosis in 76% of the cases. The lapse of time between the onset of neurological symptoms and confirmation of the diagnosis varies from 2 to 24 months, the average being 9 months. The mean age of patients suffering premonitory polyneuropathic signs of myeloma is 52 yr.

The progression of this type of polyneuropathy is often slow, death occurring, on an average, after 2 yr [4]. As in our case, therapy may bring some improvement and even remission of neurological disturbances [24].

So far, no correlation has been established between the intensity of the neuropathy and the evolution of the myeloma.

(C) *Histological aspect.* According to Henson and Urich [25] histological modifications are similar to those observed in neoplastic sensorimotor polyneuropathies. As far as we know, only one ultrastructural study of myelomatous polyneuropathies has been published [26]. Recent electron microscope studies of dysglobulinemia neuropathies concern mainly Waldenström's macroglobulinemia [6, 27–29]. In these cases, authors report that morphological modifications were compatible with remyelinating processes and that axon alterations are slight. Vital *et al.* [29] also stress that pathological lesions are closely related and proportional to clinical manifestations.

Morphological alterations in our observation, although not severe, are more consistent with the pattern of a primary demyelinating disease than with an axonal degeneration. Alterations of the walls of small arteries that we described in the nerve do not appear to have been mentioned previously. Their pathological significance is still unknown.

(D) *Electrophysiological studies.* The few electrophysiological studies in this field have all clearly demonstrated a drastic decrease of velocity in sensitive and motor nerve conduction. However, this reduction of electrical conduction has also been observed by Walsh [26] in 25% of myelomas, with no clinical evidence of neuropathy. These results tend to suggest the existence of incipient forms of dysglobulinemic neuropathies.

(E) *Pathogenesis.* Among numerous theories proposed, the most favoured pathogenic concept is now related to immunological mechanisms. The initial hypotheses originated from research by Waldenström *et al.* [30] and Zettervall *et al.* [31], who have shown that the pathological immunoglobulin (M-component) secreted by tumoral plasmocytes, has both the structure and the function of a normal antibody. Morley *et al.* [4], Bigner *et al.* [27] and Davis *et al.* [24], suggested that myelomatous cells could occasionally produce a circulating substance acting as an "antibody" against some components of the peripheral nervous system, thus inducing progressive neuropathy. Other arguments supporting immunological mechanisms are based on similarities between clinical and histological aspects of dysglobulinemic polyneuropathies and those of paraneoplastic polyneuropathies, the pathogenesis of the latter being now explained by immunological factors [32–34]. Immunofluorescence technics now yield strong arguments for this immunological theory. Our preliminary results [35] confirmed and completed by Chazot *et al.* [5], then by Propp *et al.* [6], enable us to make further comments.

(1) *Fixation site of the M-Component.* On longitudinal sections, we observe a strong fixation on the nerve sheaths, a localization already indicated by Chazot *et al.* [5], and a dense linear fluorescence in the central portion of the nerve fibres. According to Luyten *et al.* [36], such a fluorescence linear "pattern" suggests that the "antigen-antibody" interreaction occurs in the proximity of the myelin sheath. Looking at transverse sections, Chazot *et al.* [5] suggested a deposit in contact with the myelin sheaths, while Iwashita *et al.* [28] considered that deposits were between the nerve fibres. Propp *et al.* [6] even wrote that the M-Component seems directly linked to the myelin membrane. According to these assertions, the M-Component might attach itself partially on the nerve sheath, but mainly on the myelin sheaths.

(2) *Fixation mechanisms of the M-Component.* This mechanism of fixation on the peripheral nerve trunks is not clear. By proving that the M-Component of a neuropathic patient could be attached to the nerve trunk of a healthy individual, Chazot *et al.* [5] suggested that the M-Component has a specific affinity for the peripheral nerve and eventually for the myelin sheaths. However, Propp *et al.* [6] have indicated that the fixation of the M-Component on the nerve fibres is an unspecific physico-chemical reaction with no proven immunological mechanisms.

(3) *Lesion mechanisms of the M-Component.* Among the main theories, the most interesting is that of an interrelation of immunoglobulins with membrane phospholipids [37]. Fixation of the M-Component to some structures of the myelin sheath would increase myelin permeability, with

swelling then its destruction. The cytotoxic role of the activated complement system should also be considered because the third component of complement (C'3) has been found to be attached on the nerve trunks [5].

A last but tempting hypothesis is based on recent advances on amyloidosis. Glenner *et al.* [38] have well established that amyloid substance results from an insoluble and fibrillar organization of some polypeptides which, in many cases, could be immunoglobulin fragments.

According to this theory, it may be considered that M-Component deposits, observed on nerve trunks by immunofluorescent techniques represent a kind of "precursors", for the amyloid substance. The fact that these "precursors" can play a pathogenic role and induce lesions of nerve fibres, is perfectly supported by recent publications on Portuguese amyloid neuropathies. Coimbra *et al.* [39], Jorge Carvalho *et al.* [40], have demonstrated that alterations of both Schwann's cells and myelin sheaths precede the onset of amyloid deposits, these deposits subsequently aggravating the previous lesions either directly through mechanical compression or indirectly through ischemia. Immunofluorescent studies on two cases of familial amyloid neuropathies showed that amyloid substance contained an IgG with light chains type kappa [41]. Whether the immunoglobulin was a "precursor" of amyloid, or was subsequently attached on the amyloid is still to be elucidated.

To reach a unified concept between recent immunological data and results of former observations, one may advance that fixation of the M-Component in the nerve trunks is an intermediary stage or a form of transition towards the final phase of the evolution amyloidosis neuropathy.

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Importance of Schedule in Adriamycin/Cyclophosphamide Combination Chemotherapy*

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Abstract—*Use of the chemosensitive tumor* P388 *lymphocytic leukemia has facilitated the demonstration of small but perhaps important schedule-dependent therapeutic effects with adriamycin (ADR) and cyclophosphamide (CY) combination therapy. Administration of ADR at least* 3 days *pror to the administration of CY resulted in inhibition of DNA synthesis to a greater extent in tumor than in bone marrow. This preferential inhibition of thymidine incorporation into nucleic acid of tumor cells is in accord with the enhanced chemotherapeutic response observed when the agents were given in this sequence (ADR* day 5 *and CY on* day 8, 9 or 10). *This effect on response was more marked with lower doses of the drugs, which could be of clinical significance since the total dose of ADR is so restrictive.*

INTRODUCTION

IMPRESSIVE results have been obtained with the use of adriamycin (ADR) and cyclophosphamide (CY) combination chemotherapy in a number of human tumours [1–3]. In particular, the activity of this combination against carcinomas of the breast and ovary appears to be greater than expected from the single agent data available for each of these agents used alone, with responses in these malignancies at least as good as for any other drug combinations in current use [4, 5]. Previous work has demonstrated not only a definite therapeutic synergism between ADR and CY for a large number of transplantable animal tumors [6] but also a dramatic inhibition of DNA synthesis which greatly exceeded the inhibition of tumor-cell DNA synthesis caused by each agent when used alone [7]. Against the advanced murine L1210 leukemia, the activity of this combination was particularly surprising because of the relative ineffectiveness of one of the agents (ADR).

Since cumulative cardiotoxicity frequently limits continued use of ADR, often necessitating discontinuation of treatment in responsive patients, the optimal use of this valuable agent is particularly important. The present studies were designed to investigate dose and scheduling in ADR/CY combinations with a view to determining whether manipulation of either variable could improve therapeutic effect without necessarily increasing ADR dosage. Preliminary results of these studies have been reported [8].

MATERIAL AND METHODS

Adriamycin (NSC 123127) was supplied by Farmitalia, Milan, Italy and cyclophosphamide (NSC 26271) was the clinical dose form, Cytoxan®, obtained from Mead Johnson Laboratories, Evansville, Indiana. The thymidine (methyl-^{3}H) was purchased from New England Nuclear, Boston, Massachusetts and was diluted with cold thymidine (1 mg/ml) from Sigma Chemical Company, St. Louis, Missouri, yielding a specific activity of 0.45 Ci/mmole. All compounds were prepared in physiological saline and administered in a vol of 0.5 ml.

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P388 lymphocytic leukemia was serially transplanted by intraperitoneal inoculation of 10^6 ascites tumor cells into CDF_1 mice weighing 20–25 g.

In the experimental therapeutic studies, groups of 10 tumor-bearing mice were treated on day 5 with CY, followed sequentially by ADR on day 5, 6, 7, 8, 9 or 10 and conversely on day 5 with ADR followed sequentially by CY on day 5, 6, 7, 8, 9 or 10. In additon, both drugs were administered as a single agent on day 5, 6, 7, 8, 9 or 10. All agents were administered by the intraperitoneal route.

A thymidine labelling technique was used to evaluate the inhibition of DNA synthesis of tumor and bone marrow cells following drug treatment. Mice received 25 μCi of tritiated thymidine by intraperitoneal injection 15 min prior to death by cervical dislocation. In pilot studies, it was demonstrated that thymidine incorporation into DNA of tumor cells and tissues was linear between 5 and 30 min. Tumor cells and femurs were pooled in groups of 3 mice and 2–3 groups were analyzed at each time period. Tumor cells in ascitic fluid were concentrated by centrifugation (1000 × ***g***), resuspended twice in saline and counted using a Model B Coulter counter. Femurs, initially frozen in liquid nitrogen at time of death, were thawed and bone marrow was obtained by gentle aspiration in ice-cold physiological saline as previously described [7]. Nucleic acid from tumor cells and from bone marrow was precipitated by 10 vol of cold 5% trichloroacetic acid (TCA) and extracted into 2 vol of hot (90°C) 5% TCA. The extracted nucleic acid was analyzed for radioactivity in a Nuclear-Chicago Mark I scintillation counter by means of external standard channel-ratio correction and quantitated spectrophotometrically at 260 nm in a Zeiss Model PMQ II spectrophotometer. Results were expressed as disintegrations per min per optical density unit of DNA.

RESULTS

DNA synthesis in untreated tumor-bearing animals

A fall in DNA synthesis was seen in the liver, heart and bone marrow of all tumor-bearing animals over the 5- to 10-day period following i.p. implantation of 10^6 P388 cells (Fig. 1). DNA synthesis also declined in P388 cells recovered from ascitic fluid. Although absolute DNA synthesis in ascitic P388 cells was 10 times greater than that of any host tissue (in dis/min per optical density unit at 260 nm of nucleic acid), DNA synthesis fell

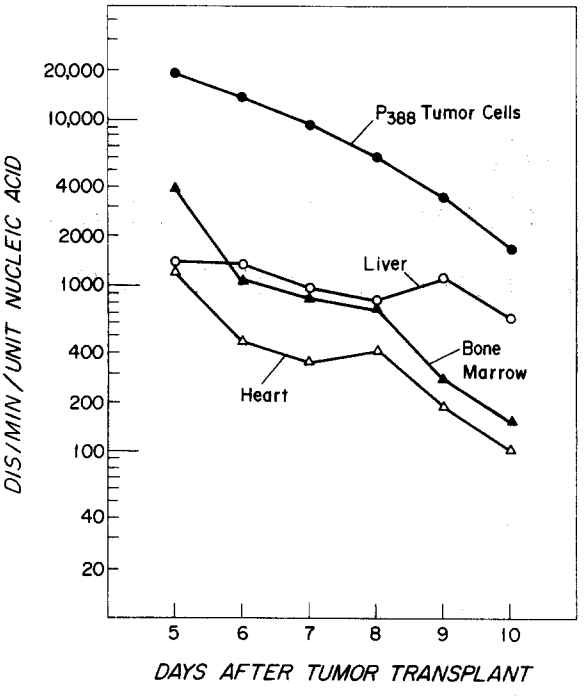

Fig. 1. Nucleic acid turnover in tissues from saline-treated, tumor-bearing mice. Tumor-bearing mice were administered 25 μCi *of tritiated thymidine* 15 min *prior to death. The DNA from tissues and P388 cells was extracted, quantitated and analyzed for radioactivity.*

equally for all tissues and P388 cells at approximately 1 log over days 5–10 (median day of death of untreated tumor-bearing animals was day 11).

Survival of tumor-bearing animals treated with ADR/CY combination

Treatment of P388 leukemia with ADR or CY in combination resulted in an increased life span (ILS) greater than that expected from the summation of each drug given individually in all treatment groups (Fig. 2). Maximal ILS with single-agent treatment was 50% for ADR (8 mg/kg given once on day 5 after tumor inoculation) and 120–140% for CY (200 mg/kg given once on day 5, 6 or 8). With combination treatment, several interesting trends were apparent. The administration of a high dose of cyclophosphamide with adriamycin resulted in maximum ILS of over 300% and in a significant number of long-term survivors (>60 days) when administered on day 5. This advantage was decreased progressively when adriamycin was withheld until day 6, 7 or 8. The administration of adriamycin followed by cyclophosphamide showed somewhat less increase in ILS as well as in long-term survivors on day 5 and decreased thereafter until day 8. The administration of adriamycin first followed by cyclophosphamide on day 8, 9 or 10 resulted in a rebound in both ILS %

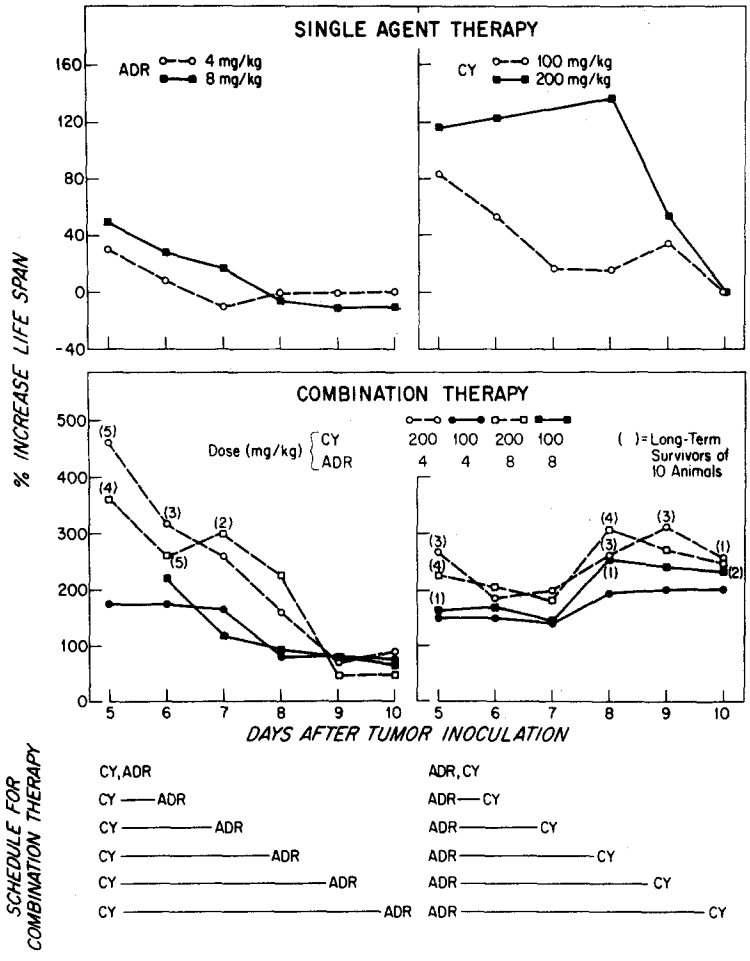

Fig. 2. Effectiveness of sequential therapy schedules with ADR and CY against P388 leukemia as opposed to each agent given singly. 10^6 *P388 cells were inoculated i.p. on* day 0 *and drugs were administered i.p. on* day 5 *through* 10 *according to schedule for combination therapy, or as a single dose for adriamycin or cyclophosphamide on* day 5, 6, 7, 8, 9 *or* 10. *Numbers in parentheses indicate number of long-term survivors* (>60 days) *out of* 10 *animals.*

and long-term survivors, which greatly contrasts with the results of administering cyclophosphamide first followed by adriamycin on day 8, 9 or 10.

The single agent therapy data indicated that cyclophosphamide was a better chemotherapeutic drug than adriamycin. This finding also appears to be true in combination therapy. Cyclophosphamide administered first, followed by adriamycin, showed maximum ILS % on day 5 but showed a progressive decrease thereafter. When adriamycin was administered first followed by cyclophosphamide, there was a marked effect of cyclophosphamide, especially on days 8 through 10. In the subsequent studies, we have tried to evaluate the relationship among schedules of administering the two agents, tumor burden and inhibition of DNA in both tumor cells and bone marrow.

Tumor cell burden following treatment with ADR/CY

Total ascitic cell counts fell rapidly after treatment with ADR or CY (Fig. 3). Sham injection with saline did not affect the exponential proliferation of i.p. implanted P388 cells (Fig. 3A), which increased by almost 1 log over days 5 through 10; CY at 250 mg/kg had a greater cytotoxic effect than ADR at 10 mg/kg when given as a single agent on day 5 after tumor implantation (2 log vs 1.5 log, respectively, Fig. 3B), but both agents were relatively less effective when given singly on day 8 (*ca.* 1 log, Fig. 3C). When ADR and CY were given in combination, they were not more cytotoxic than CY when given alone on day 5 (Fig. 3D). Similarly, when ADR and CY were given with a 3-day separation (irrespective of which was given first), they were at least as cytotoxic as when both agents were given on day 5, despite delaying one of the agents for an additional 3 days (Fig. 3D). Thus, the tumor burden did not appear to be decreased as a result of administering the two agents in combination.

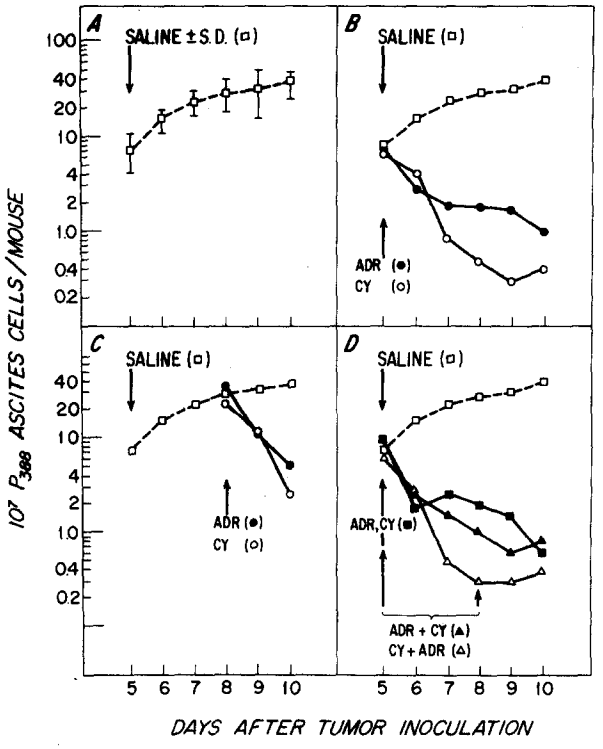

Fig. 3. Effect of ADR and CY on P388 cells growth. 10^6 *P388 cells were inoculated i.p. on* day 0 *and drugs (ADR,* 10 mg/kg; *CY,* 250 mg/kg) *or saline were administered i.p. according to the schedule shown in the figure.*

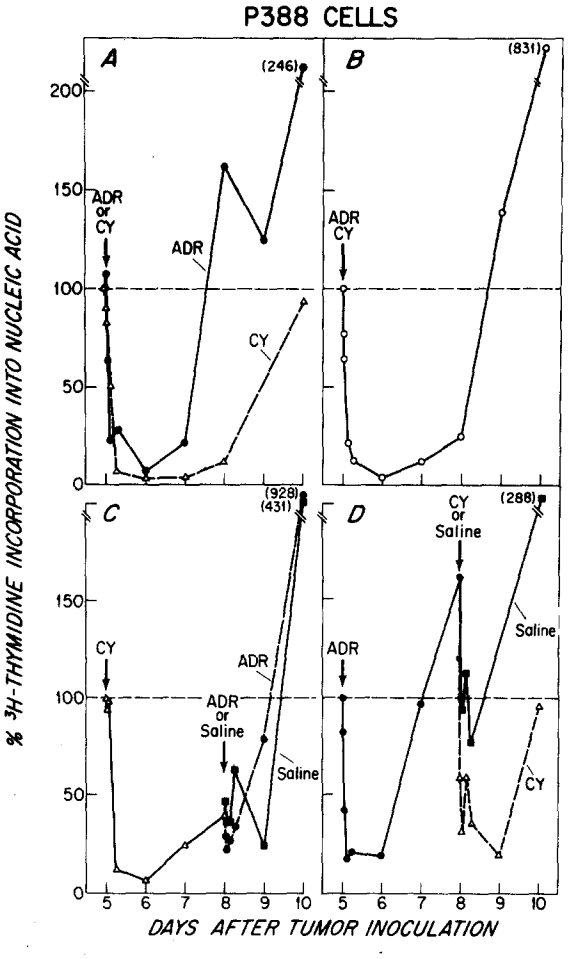

Fig. 4. Effect of ADR and CY on the percent incorporation of ^{3}*H-thymidine into nucleic acid of P388 cells of tumor-bearing mice.* 10^6 *P388 cells were inoculated i.p. on* day 0 *and drugs (ADR,* 10 mg/kg: *CY* 250 mg/kg) *or saline were administered i.p. according to the schedule shown in the figure.*

Inhibition of DNA synthesis in tumor cells and bone marrow

Both ADR and CY inhibited DNA synthesis in P388 tumor cells (Fig. 4A); extent and duration of this inhibition were greater for CY than for ADR and recovery from the CY-induced inhibition began later than recovery after ADR. Simultaneous administration (day 5) of ADR and CY did not inhibit DNA synthesis more effectively than CY alone (Fig. 4B). ADR given on day 8 (after CY on day 5) did not result in further effective inhibition of DNA synthesis (Fig. 4C); at 10 days, tumor cell DNA synthesis was 430% of control. However, when ADR was given *before* CY (Fig. 4D), the ADR-induced inhibition of DNA synthesis (day 5) was followed by an equally pronounced CY-induced inhibition (day 8). Subsequently, at day 10, tumor cell DNA synthesis had just recovered to 100% of control with no overshoot.

The effect of ADR and CY on DNA synthesis in bone marrow (Fig. 5) differed from that for P388 cells in the same animal (Fig. 4). Briefly, the depth and duration of this inhibition and the recovery time for both drugs were comparable (Fig. 5A). In comparing bone marrow with tumor cells, there is a marked difference in the duration of the inhibition as well as the magnitude of the overshoot depending upon whether the drug was used singly (Figs. 5A and 4A) or in combination on day 5 (Figs. 5B and 4B). When CY was administered on day 5 and ADR on day 8. ADR had little effect on the overshoot in the incorporation of ^{3}H-thymidine into DNA of bone marrow (Fig. 5C) being similar to that for P388 cells (Fig. 4C). The magnitude of the inhibition of DNA synthesis of bone marrow, compared to tumor cells, was most striking when ADR was given on day 5 and CY on day 8 (Figs. 5D and 4D). The inhibition of ADR-induced overshoot by CY in bone marrow did not decrease the ^{3}H-thymidine incorporation to less than 400% of control, whereas in P388 tumor cells the inhibition was decreased to about 20% of control. Further, the recovery of DNA synthesis in bone marrow was greater than 1000% of control on day 10, whereas in P388 cells, the recovery was equivalent to control with no overshoot at the same time period.

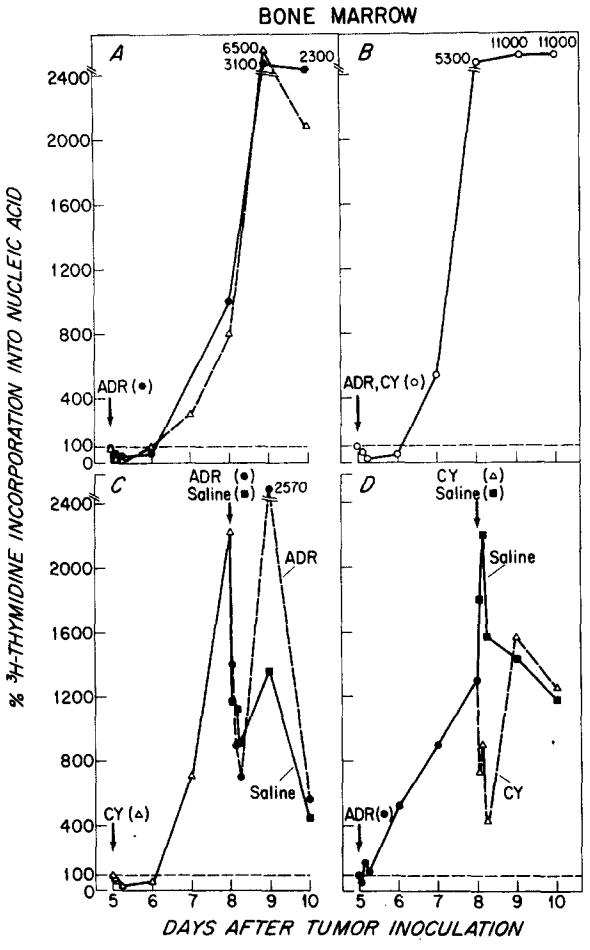

Fig. 5. Effect of ADR and CY on the percent incorporation of ^{3}H-thymidine into nucleic acid of bone marrow of P388 tumor-bearing mice. The 100% incorporation represents the nucleic acid turnover in bone marrow as seen in Fig. 1.

DISCUSSION

Encouraging preliminary results in the treatment of carcinoma of the breast (4), ovary (5) and uterine endometrium (3) with ADR/CY combination chemotherapy have been paralleled by the demonstration of therapeutic synergism in animal tumor systems. However, the optimal dose, timing and schedule of administration of this combination are not known. In L1210 leukemia, a steep dose–response curve was obtained for both agents in combination (7). Separation of the agents for periods up to 24 hr did not appear to exert a clear therapeutic advantage. In the present studies, use of a more chemosensitive tumor has facilitated the demonstration of small but perhaps important therapeutic differences with schedule alterations.

The most striking schedule-dependent effect appears to be the superiority of ADR→CY combinations over CY→ADR combinations. Even when sequential treatment with CY was delayed to day 10 (advanced disease, one day before death), long-term survivors were occasionally seen (Fig. 2). By contrast, treatment with CY on day 5 and delay of ADR until day 9 or 10 gave results no better than CY treatment only on day 5. Since ADR is a less effective agent than CY in P388 leukemia, this observation was particularly unexpected. The ability of CY to produce a real inhibition of tumor cell DNA synthesis on day 8 after ADR (given on day 5) further reflects the enhanced effectiveness of this sequence, since ADR given on day 8 (after CY on day 5) failed to inhibit further DNA synthesis in tumor cells. The observations of transient, reversible inhibition of bone marrow DNA synthesis are in accord with the known myelosuppressive effects of this combination in man.

The explanation for these observations is unclear. It has previously been postulated that the alkylating activity of CY may be enhanced by ADR due to increased receptivity of the ADR-damaged DNA molecule [7]. Intercalation of ADR into DNA appears to be an important mechanism of its action and it is possible that CY effectiveness is most pronounced when partial recovery from the DNA-intercalation action of ADR is taking place. Increased sensitivity to the effect of an alkylating agent might reasonably be expected during this period. It has been shown that ADR inhibits the hepatic mono-oxygenase system [9]. CY depends on hepatic enzymes for activation [10]. Phenobarbital, which increases the mono-oxygenase system, stimulates leukopenia rather than chemotherapy by CY, whereas SKF-525A, which inhibits the mono-oxygenase system, decreases leukopenia and enhances chemotherapy by CY [11]. ADR may act similarly to SKF-525A. The possibility of increased circulating levels of activated CY or of ADR, when the drugs are given in combination rather than singly, has been investigated and does not appear to be a likely explanation [7]. Finally, the liver may normally represent a protected compartment from the action of CY. If ADR acts directly on hepatocytes, influx of activated CY may be more effective in the presence of ADR, thus allowing for greater clearing of tumor-cell infiltration in the liver, itself a possible cause of death in experimental leukemias.

The importance of proper scheduling has been increasingly stressed since the emergence of more effective combination chemotherapy for human leukemias and solid tumors [12]. The combinations of vinblastine and bleomycin [13] and methotrexate and L-asparaginase [14] are good examples of schedule-dependent pairs

of drugs. Proper scheduling with ADR and CY is of crucial importance for clinical cancer chemotherapy not only in order to ensure maximal antitumor effect but also to conserve ADR for each patient as long as possible; 550 mg/m^2 is at present the total recommended cumulative dose [15]. Observations regarding schedule-dependency may also shed light on the mechanism of action of these agents and, in particular, on their unusual degree of therapeutic synergism.

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A Fetal Antigen in a Mouse Fibrosarcoma with Possible Cross-reactivity with an Adult Mouse Skin Component*

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Abstract—*A late gestation–neonatal phase antigen has been found in a mouse fibrosarcoma. The cross-reacting antigen of tumor, fetal and neonatal mice possessed gamma-mobility upon immunoelectrophoresis in* 2% *agar at* pH 8.6 *and was undetectable in extracts of normal adult internal organs. A related, unstable, constituent was detected in normal adult mouse skin.*

INTRODUCTION

ONE OF the characteristics of cancer tissue is its frequent association with embryonal antigens [1–6]. In a continuation of the study of such antigens in our laboratory [3], rabbit antisera prepared to aqueous extracts of a 3-methylcholanthrene-induced mouse fibrosarcoma were found, after appropriate absorption, to precipitate an antigen common to the tumor, late gestation stage mouse fetuses and neonatal mice. This report describes these findings.

MATERIAL AND METHODS

The mouse tumor cell line "QUA", cloned from a 3-methylcholanthrene-induced C57 BL/6 mouse fibrosarcoma, was kindly furnished by Dr. June L. Biedler of the Memorial Sloan-Kettering Cancer Center [7]. Tumors arising in several C57 BL/6 mice after inoculation of QUA cells were pooled and extracted with phosphate-buffered saline (PBS) as described by Stonehill and Bendich [8]. Another 3-methylcholanthrene-induced mouse fibrosarcoma, MeTu, in second passage, was kindly furnished by Dr. Ellen Borenfreund of this laboratory. The age of C57 BL/6 mouse fetuses was determined by mean crown–rump length [9]. Fetal and neonate trunk portions (body, less skin and limbs) and fetal organs were also extracted with PBS. Antisera were prepared in New Zealand white rabbits by 3 weekly subcutaneous inoculations of 5 mg of lyophilized PBS extracts of QUA tumor tissue using the immunization procedure of Stonehill and Bendich [8]. Pooled antisera were absorbed with normal adult mouse serum and with lyophilized PBS extracts of adult mouse internal organs until all precipitin activity to adult serum and adult internal tissue extracts was eliminated. Immunoelectrophoresis was carried out for 45 min at 25 mA/200 V in 2% agar using barbiturate buffer, pH 8.6, of 0.075 ionic strength (Beckman B-2 buffer, Beckman Instruments, Inc., Palo Alto, CA). For single radial immunodiffusion assay [10], 2.5 ml of 1% agarose (w/v) in Beckman B-2 buffer, pH 8.6, was mixed with 100 μl of rabbit pre-immunization serum and 100 μl of adult internal tissue-absorbed anti-QUA tumor serum and layered on a 1 × 3 in. microscope slide. Antigen wells, 3.75 mm dia, were cut and filled with 10 μl of antigen solution previously adjusted to a concentration of 2–4 mg protein/ml [11]. Slides were incubated at 4°C for 14 days, stained [12] and the diameters of the precipitin discs measured. The radial immunodiffusion unit employed is the square of the diameter (in mm) of the precipitin disc minus the square of the antigen well diameter per microgram of protein assayed.

RESULTS

When adult internal tissue-absorbed anti-

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QUA tumor antiserum was assayed by Ouchterlony double-diffusion, a precipitin line of identity was obtained when tumor and embryo extracts were in adjacent wells, Fig. 1(a). This line bent slightly away from an adjoining well when adult skin extracts were examined in the same plate indicating the possible occurrence of cross-reacting determinant(s) in adult mouse skin. This finding is reminiscent of the presence of a fetal-related antigen in normal adult human skin cell cultures which was detected by

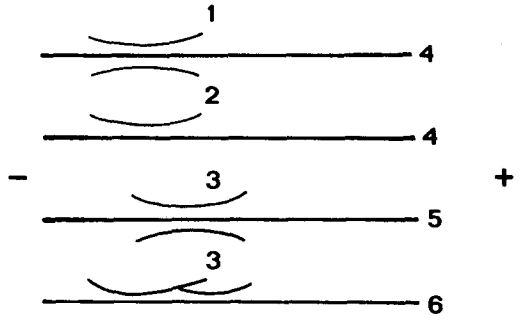


Fig. 2. Demonstration of a cross-reacting antigen in a mouse fibrosarcoma and mouse embryo employing agar immunoelectrophoresis; 25 mA/200V *for* 45 min, *barbitol buffer,* pH 8.6, *ionic strength* 0.075. (1) *Extract of* 17-day *skinned fetuses;* 90 *μg of protein placed in well;* (2) *Extract of QUA tumor;* 10 *μg of protein placed in well;* (3) *Pooled whole adult C57 BL/6 mouse serum;* 10 *μl placed in well;* (4) *Adult internal organ-absorbed rabbit anti-QUA tumor antiserum;* (5) *Rabbit anti-mouse* $\beta_1 C$ *globulin antiserum;* (6) *Rabbit antiserum to mouse IgG.*

immunofluoroscence techniques using serum from sarcoma-bearing patients [13]. Moreover, skin cells have been shown to afford immunoprotection against transplantation challenge with QUA [14], and cross-reactivity among mouse fetus, mouse tumor and adult mouse skin has been reported [3, 15]. These data suggest the presence of a fetal-type antigen in normal adult skin. The appearance of fetal antigen in fragments of normal skin after 9 days in culture [16] is consistent with this interpretation. A precipitin line of identity was produced when the following were tested with adult internal organ-absorbed anti-QUA tumor serum, Fig. 1(b): extracts of 15–19 day skinned fetuses or skinned postpartum mice, extracts of an independent 3-methylcholanthrene-induced fibrosarcoma, MeTu, pooled sera of QUA tumor-bearing mice, and sera of animals carrying the MeTu tumor. Adult serum and extracts of normal adult internal organs were negative, Fig. 1(a). Absorption of the anti-QUA tumor antiserum with lyophilized extracts of adult skin, 17-day skinned fetus, or QUA tumor completely removed precipitin activity to all three.

Immunoelectrophoresis disclosed a single precipitin arc with gamma mobility when embryo and tumor extracts were tested with adult internal organ-absorbed anti-QUA serum (Fig. 2). The cross-reacting antigen of normal skin was not seen in the usual 45 min immunoelectrophoretic analysis. However, a precipitin arc of apparent zero mobility was observed at much shorter times (5–10 min). Its absence after longer times of electrophoresis suggests an innate instability and further studies of

Table 1. Quantitation of gamma-antigen in saline soluble extracts of mouse tissues by single radial immunodiffusion assay

Tissue extracted	Radial immunodiffusion units*
Fetal trunks†	
day 15	0
day 16	11.2±1.4
day 17	14.3±2.6
day 18	11.0±0.7
day 19	14.1±1.5
Neonates‡	
newborns	19.5±1.6
1 day postpartum	9.4±0.8
2 day postpartum	7.2±0.7
3 day postpartum	0
Neonatal organs§	
spleen	191.8±23.8
thymus	24.6±2.8
liver	10.0±1.4
brain	0
intestine	0
kidney	0
lung	11.3±1.4
Adult organs	
viscera‖	0
muscle	0
Tumor	
QUA	43.4±5.2

*Radial immunodiffusion unit is defined as the square of the diameter (in mm) of the precipitin disc minus the square of the diameter of the antigen well per microgram of protein assayed.

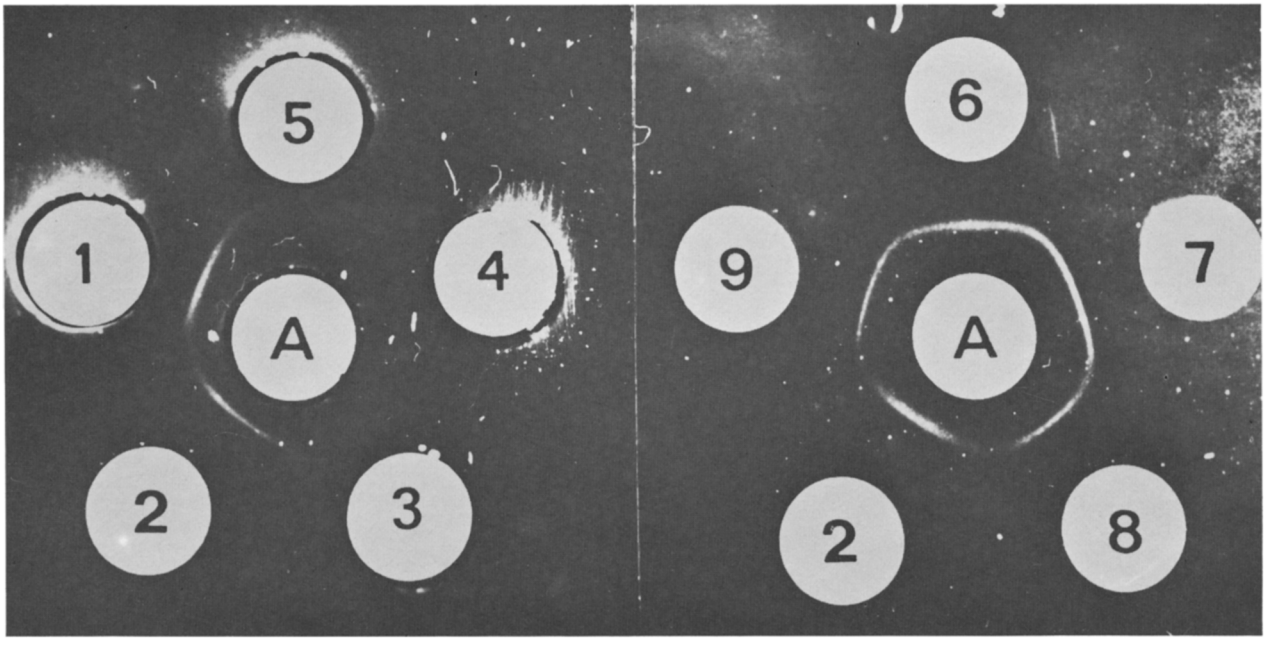
†Saline extracts of fetal trunks as in ‡ (15–19th day of gestation).

‡Saline extracts of trunk portions of newborn mice (less head, appendages and skin).

§Organs removed from newborn animals immediately after parturition.

‖Saline extracts of heart, lung, liver, spleen and kidneys of adult mice.

These data represent the average of 8–10 individual assays. Antigen preparations which failed to yield precipitin discs were further assayed at both higher and lower antiserum dilutions, as well as at various antigen concentrations, to eliminate the possibility of false negatives.



(a) (b)

Fig. 1. (a) *Demonstration of cross-reactivity between embryo and tumor antigens using Ouchterlony double-diffusion precipitin reaction in agar gels (Hyland Labs, D pattern). A. Absorbed anti-QUA antiserum in center well.* 1. *Extract of pooled* 17-day C57 *BL/6 skinned fetuses* (40 μg *protein); 2. QUA tumor extract* (20 μg *protein); 3.* 5 μl *adult* C57 *BL/6 mouse serum; 4. Extract of pooled adult* C57 *BL/6 mouse internal tissue* (40 μg *protein); 5. Extract of normal adult* C57 *BL/6 mouse skin* (40 μg *protein).* (b) 6. *Extract of* 1-day *postpartum skinned mice* (40 μg *protein); 7. Extract of an independent* 3*-methyl-cholanthrene-induced fibrosarcoma, Me Tu* (20 μg *protein); 8.* 5 μl *pooled serum from the Me Tu-bearing mice;* 9. 5 μl *pooled serum from QUA tumor-bearing mice.*

the skin antigen are in progress (P. J. Higgins, personal communication).

Quantitative evaluations of the concentration of gamma-antigen in various mouse tissues by radial immunodiffusion assay are shown in Table 1. The gamma-antigen appeared in fetal tissues after day 15 of gestation and persisted until 2 days postpartum. Since the concentration was somewhat higher at parturition, the distribution of the antigen at this stage was determined for several organs. A high concentration occurred in the spleen, while the levels in thymus and liver were similar to that in whole trunk extracts. The gamma-component was undetectable in extracts of brain, intestine and kidney. The significance of this unequal distribution is not apparent, although it may be that the fetal/neonatal spleen is the principal site of gamma-antigen biosynthesis.

DISCUSSION

The antigen described here appears to be analogous to the gamma-fetoprotein in human neoplasms described by Edynak *et al.* [2] who used sera from cancer patients for its detection in their studies. However, these authors [2] did not detect antigens with gamma mobility in fetal mouse serum nor in the 20 spontaneous mouse mammary tumors examined.

The presence of gamma-antigen could not be ascertained in extracts of QUA tumor cells which had been propagated *in vitro* with the immunological assays employed here. However, upon re-inoculation into animals, these cells grew into tumors from which the gamma-antigen could be extracted [17]. This loss and regain of antigen could represent the net result of an interaction of the tumor with its environment, further understanding of which may shed light on the general problem of tumor-host relationships. It may also imply perhaps a cessation of the synthesis of the fetal component when tumor cells are propagated in culture. Alternatively, the gamma-component may not be synthesized in the tumor *in vivo* but may be acquired from another tissue source via the blood and processed in the tumor.

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The Relationship of Thymidine Metabolism to the Use of Fractional Incorporation as a Measure of DNA Synthesis and Tissue Proliferation

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Abstract—*After* 1 hr, *metabolism of thymidine*-6-3*H* (*TdR*-3*H*) *in tumour-bearing August female rats yielded a number of radioactive products. Rapidly dividing tissues (tumour, gut and spleen) incorporated high levels of tritium into DNA as labelled thymine. Conversely, the excretory organs (liver and kidney) had high levels of labelled catabolites, which included, tritiated water* (^3H_2O), *beta-amino isobutyric acid* (*βAIB*) *and beta-ureidoisobutyric acid* (*βUIB*). *Other metabolites were incorporated irreversibly into cellular RNA, proteins and lipids and others were in a form still amenable for conversion to DNA-thymine.*

Fractional incorporation (*F.I.*) *reflects the rate of DNA synthesis within an individual tissue by expressing the DNA uptake of TdR-3H as a function of the total tissue tritium. In the spleen the F.I. response following a single dose of cyclophosphamide* (*CY*) *was shown to correlate with gross morphology and DNA content. Similarly the histological response of the jejunal mucosa after a single dose of methotrexate* (*MTX*) *correlated with F.I. data for TdR-3H and deoxyuridine*-6-3*H* (*UdR*-3*H*).

INTRODUCTION

THE INCORPORATION of tritiated thymidine (TdR-3H) into DNA has long been used as a measure of the rate of DNA synthesis. Possibly the most complete review of thymidine usage is by Cleaver [1]. In any labelled precursor study it is important that the molecule should reach the cell in a chemical form that still resembles the parent compound. Any metabolism may alter the chemical nature of the labelled compound and thus limit its availability. In this context, Wand *et al.* [2] have shown that self-radiolysis of TdR-3H can result in TdR breakdown and for this reason these studies have utilised only freshly prepared TdR-3H. Even so *in vivo* metabolism of TdR has been shown to give products which are available for the synthesis of macromolecules other than DNA (Fig. 1). In rat liver more than 30% of the tritium from TdR-methyl-3H was incorporated into protein, RNA and lipid material [3]. Although a tritium atom attached to the methyl group of TdR is probably more labile than a tritium atom on C 6, it was considered probable that levels of tritium from TdR-6-3H would become associated with extraneous macromolecules. Such extraneous incorporation is obviously important in consideration of the rate of DNA synthesis as measured by TdR-3H incorporation into DNA, since they may limit the availability of the precursor for DNA synthesis. Because of this, the catabolism of TdR-6-3H (Fig. 2) has been studied by measuring the activity associated with the various cellular fractions of a number of rat tissues, after a 1 hr pulse label of freshly obtained TdR-6-3H.

The parameter of Fractional Incorporation (F.I.) which we have made use of in previous studies [4–6], expresses the incorporation of TdR-3H into DNA as a fraction of total cellular tritium. This measure has been shown to be a valuable alternative to specific activity (DPM 3H/mg DNA) in correlating levels of DNA synthesis with tumour cell kinetics in mice

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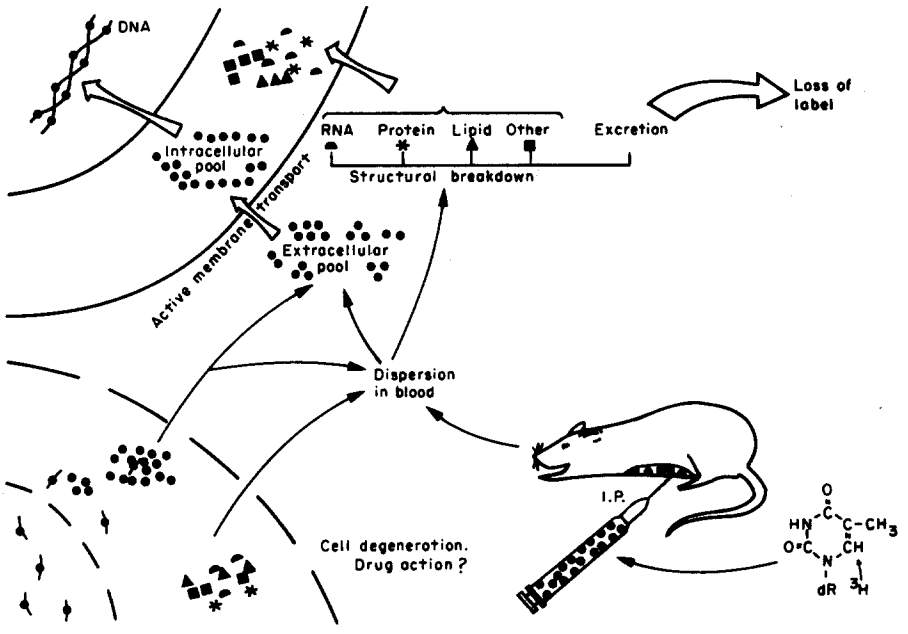


Fig. 1. The possible metabolic fate of thymidine-6-3H.

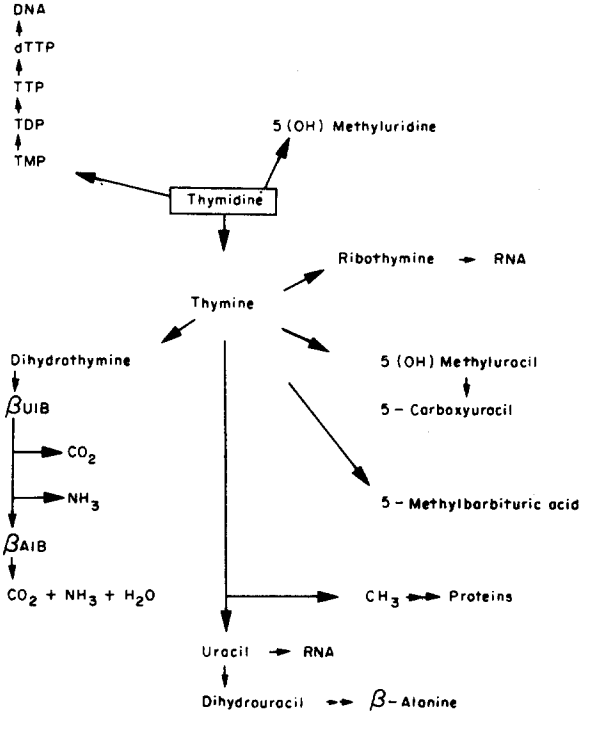


Fig. 2. A simplified version of the possible metabolism of thymidine. Pathways on the right are minor and may only occur in some bacterial and mammalian cells.

[7] especially after chemotherapy. This present study considers how the chemical state of the 3H label in the acid soluble fraction could affect F.I. measurements and also relates F.I. values to other modes of measuring tissue proliferation following cytotoxic drug therapy.

MATERIAL AND METHODS

The animals used were 5–6 month old inbred female August rats bearing s.c. A15 carcinomata. Cyclophosphamide (WB Pharmaceuticals Ltd., Bracknell, Berks) and

methotrexate (Lederle Laboratories Ltd.) were administered intraperitoneally to lightly anaesthetised rats. Thymidine-6-3H, 24 Ci/mmole, deoxyuridine-6-3H, 21 Ci/mmole (Radiochemical Centre, Amersham) were administered 1 hr prior to death by cervical dislocation. Excised tissues were treated biochemically and histologically as described previously [5]. DNA was estimated colourimetrically [8] after extraction using a modification of the Schmidt–Thannhauser procedure [9].

The following scheme was undertaken to further characterise the acid soluble constituents from tissue samples:

15 ml Acid Soluble Extract → Count 1 ml → A

5 ml lyophilise. Redissolve in 5 ml dH_2O. Count 1 ml. Calculate ^3H_2O. → B		5 ml mix with 100 mg acid washed, neutralised charcoal [10]. Leave 1 hr at room temperature with intermittent mixing. Centrifuge 2000 rev/min during 5 min.
	C ←	Decant supernatant and count aliquot. Wash precipitate with 2 × 5 ml dH_2O. Count resultant washings to check for residual 3H. Add 10 ml ethanol/NH_3/dH_2O solution (2 : 1 : 2) to charcoal and mix. Leave overnight at 37°C. Centrifuge to clarify, 2500 rev/min during 15 min. Decant and evaporate to dryness in stream of warm air. Redissolve in 0.5 ml dH_2O.
	D ←	Count an aliquot and carry out thin layer chromatography.
Count from RNA fraction		E
Count from DNA fraction		F
Count from protein fraction		G
Total 3H in fraction		A + E + F + G = H

The metabolic fate of the tritium from TdR-3H may be calculated thus:

% of DNA incorporated thymine	= F/H × 100
% of ribothymine in RNA	= E/H × 100
% of amino acid 3H	= G/H × 100
% of ^3H_2O	= B/H × 100
% of non-nucleotide 3H	= C/H × 100
% of acid soluble 3H	= A/H × 100

Thin layer chromatography of thymidine breakdown products

For all chromatographic separation procedures TLC, Ready Plastic Sheets F1140 PEI/LS 254 PEI cellulose with luminescer (254 nm), 20 × 20 (Schleicher and Schull, Dassel, West Germany) were used.

The method used was similar to that of Randerath and Randerath [11]. Ten microlitres of each sample, together with prepared standard solutions, were applied to a thin pencil line drawn horizontally 2 cm from the base of the plate. Duplicate plates were run in the following solvent systems:

1. 1 N acetic acid run to 2 cm above the origin, then immediate transfer without intermediate drying to 1 N acetic acid and 3 M lithium chloride (9 : 1). Results obtained using

standard solutions gave the following R_F values:

TdR	0.74
T	0.68
TMP	0.42
TDP	0.12
TTP	0.099

These values were in accord with the theory that separation is dependent upon the phosphate moieties of the nucleotides.

2. Fink [12] has outlined a number of the less common catabolites of thymine-14C. To test for the presence of these after TdR-3H, a solvent of tertiary-butyl alcohol, methylethyl ketone, water and ammonium hydroxide (40:30:20:10) was employed using Whatman No. 3 chromatography paper. A few of the possible thymidine catabolites are listed below with their R_F values [12]:

Thymidine	0.58
Thymine	0.55
βAIB	0.33
βUIB	0.34
Dihydrothymine	0.78

After development chromatograms were dried in a stream of warm air and cut horizontally into 1 cm strips, so that with a solvent front 15 cm above the origin, 17 strips were cut (allowing overlap at both ends).

These strips were placed in glass scintillation vials and eluted with 1 ml of 1 N PCA at room temperature for 1 hr. After this, 10 ml of scintillant [7 vol of butyl-PBD (0.6% w/v) in toluene plus 3 vol tergitol TP9] was added and counting was carried out as previously described [5].

Preliminary experiments were undertaken to check the possibility of loss of counts through quenching by either the cellulose or paper strips. A known amount of tritium was counted in the presence and absence of both PEI cellulose and paper. Counts for all samples were comparable, suggesting no loss in efficiency through particle adsorption of radioactivity. In addition, total recovery of the original spotted radioactivity was achieved.

Estimation of fractional incorporation

Values for F.I. of 3H-TdR and 3H-UdR were calculated from the following formula:

$$\text{F.I.} = \frac{^3\text{H dis/min in DNA}}{^3\text{H dis/min in total tissue}}.$$

RESULTS AND DISCUSSION

Products of thymidine-3H metabolism

The proportions of the total tritium fractions isolated from liver, kidney, tumour, gut and spleen at 1 hr after injection of TdR-3H are shown in Table 1.

The TLC method used did not allow identification of all catabolites, thus there is a line in Table 1 which represents unknown compounds. The suggestion by Steel and Lamerton [13] that after 1 hr all tritium from TdR-3H was localised either in DNA or in tritiated water (^3H_2O) was shown not to be the case in these tissues. Certainly in the rapidly dividing tissues (tumour, jejunal mucosa and spleen) much of the label was localised as thymine-3H in DNA. However, substantial amounts of label were also associated with other macromolecules and metabolites. The proportion of label in the various metabolites was variable and dependent upon the tissue. The highest proportions of labelled β-amino isobutyric (βAIB) and β-ureido iso-

Table 1. The incorporation of 3H from 6-3H-TdR into cell macromolecules

Fraction \ Tissue	Liver	Kidney	Tumour	Gut	Spleen
dTTP in DNA	2.0	2.5	57	62	82
RNA	2.5	4.5	1.0	6.5	1.0
Protein + Lipids	6.5	6.0	1.0	4.0	1.0
^3H_2O	15.0	35.0	12.5	15.0	9.5
Unchanged TdR	0.5	1.0	4.0	2.5	1.0
*Thymine	12.0	14.0	6.5	4.0	2.0
βAIB and βUIB	54.0	32.0	10.5	1.5	1.5
Nucleotides TMP, TDP, TTP	4.0	3.5	2.5	1.5	1.5
Unidentified components	3.5	1.5	5.0	3.0	1.0

*Dihydrothymine may be a possible contaminant of the thymine fraction. Values are expressed % of total extractable 3H.

butyric acids (βUIB) were found in the liver and kidney. In the liver of male Lewis strain rats, Chang and Looney [14] found that up to 66% of the 3H from TdR-3H was present in βAIB. The results presented here show a slightly lower, approximately 54%, incorporation of 3H from TdR. The high levels of these non-protein amino acids in the liver and kidney were not surprising in view of the excretory function of the organs. The tumour showed significant amounts of βAIB. Similarly, patients suffering from various types of lymphoma have been shown to excrete quantities of these acids after administration of nitrogen mustard [15]. Also, patients fed diets rich in DNA have been shown to excrete large amounts of βAIB [16]. These facts when considered in relation to the catabolic pathway of thymidine would suggest strongly that the non-protein amino acids play no role in the anabolic synthesis of cell macromolecules. Therefore the label localised in such metabolites will no longer be available for DNA synthesis.

Tritiated water contributed between 10 and 20% of the total tissue 3H in the liver, tumour, intestinal mucosa and spleen. Again, not unexpectedly, the kidney had the highest level, 35%. Presumably the tritiated water reaches equilibrium with the other tissue fluids and plays little significant part in the anabolic synthesis of DNA.

Localization of label in RNA, protein and lipids was not as high as that found with Me-3H-thymidine [3]. Liver, kidney and jejunal mucosa had approximately 10% of the label associated with these molecules whereas tumour and spleen had only 2%. This tritium, once again, is presumably not available for DNA synthesis.

Contrary to the results discussed so far, the tritium associated with nucleotides may well be utilizable for DNA synthesis. Acid soluble dTDP has been shown to be a direct progenitor of DNA thymidylate [17], hence the low levels of nucleotide-associated tritium may well be involved in the DNA synthetic pathway.

Two pathways of thymine utilisation, one synthetic and the other degradative, are known to be functional in all tissues, their relative activities depending upon the physiologic state of the tissues. In the synthetic pathway, thymine is converted to thymidine by attachment of a deoxyribose moiety and this is phosphorylated by thymidine kinase to form thymidylic acid. Thymidylic acid is then further phosphorylated to dTTP which is utilised for DNA synthesis. Studies in bacterial cells in cultivated Novikoff hepatoma cells have suggested a compartmentalisation of thymine and thymidine with both being used as direct DNA precursors [18–20]. In addition, it has been suggested that thymine incorporation into the DNA of various rat liver hepatomas may be the result of either the deamination of 5-methylcytosines or the recycling of thymine from RNA turnover [21]. Judging from these findings it appears possible that the thymine found in these tissues may be available for DNA synthesis.

Thus there appears to be a balance between labelled molecules, some of which are only catabolites e.g. ^3H_2O, βAIB, βUIB, some of which cannot be incorporated into DNA since they are irreversibly associated with RNA, proteins and lipids and finally those which may still be metabolised to DNA-thymidylate; e.g. unchanged thymidine, nucleotides and possibly thymine.

In general these results indicate that after a 1 hr pulse label little of the TdR-3H remains in an unaltered form. In the fast-dividing tissues, spleen, gut and tumour the majority is incorporated into DNA via dTTP. In these tissues very low levels of tritiated metabolites are in a form amenable for further metabolism to DNA. The liver and kidney have low levels of incorporation into DNA and high levels of labelled catabolic products.

Comparison of specific activity and fractional incorporation at variable thymidine doses

By increasing the radioactive dosage of TdR-3H, theoretically more labelled TdR should enter the *in vivo* pool and eventually become incorporated into DNA. Figure 3 shows that using a radioactive dose range of 0.5–2.5 mCi/kg the increase in tissue acid soluble and total tritium was linear after a 1 hr pulse label. No attempt was made to dilute the TdR-3H with unlabelled TdR to give a standard injected mass, since the dilution of a "labelled-bolus" of TdR-3H when injected is considerable. For the dose range of TdR used, every molecule of TdR-3H is diluted by 10^7–10^8 unlabelled molecules in the animal. This linear increase in the cellular pools will continue until a saturation-state is reached. The acid soluble pool levels of TdR-3H in liver are the highest. The major part of this tritium is in catabolite form, as is the acid soluble tritium from the other tissues. The differences between acid soluble and total levels of tritium represent uptake into macromolecules (DNA, RNA and protein). A plot of the specific activity (counts/min/mg DNA) for the same samples as shown in Fig. 3 revealed a linear increase similar to that of the acid soluble and total cell pools. Figure 4(a) shows that the actively dividing tissues, gut, spleen and tumour have the steepest gradients.

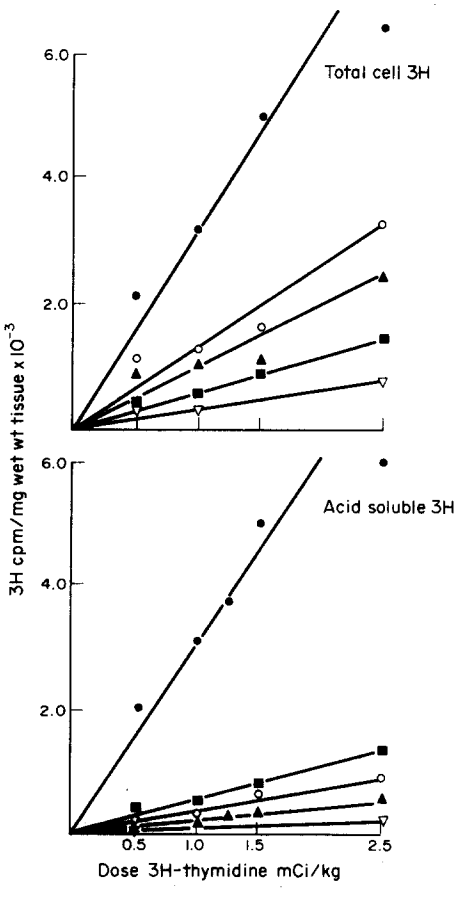


Fig. 3. Total and acid soluble 3H levels in tissues 1 hr *after variable doses of thymidine-6-3H.*
●—*liver;* ■—*kidney;* ○—*gut;* ▲—*spleen;* ▽—*tumour. The ordinate is the* counts/min *wet weight tissue* $\times 10^{-3}$. *The abscissa is the dose of thymidine-6-3H in* mCi/kg. *Each point is the mean of* 5 *experiments. Standard errors are less than* 10%.

The fact that no shoulders were observed on the curves suggested that the precursor pools did not contain sufficient labelled molecules to saturate the thymidylate pool at the dose-range used.

The F.I. data, Fig. 4(b), shows a different pattern over the same dose range. There was no such linear increase as with specific activity. Instead the fractional incorporation values remained more or less similar. This would agree with the predicted pattern for the fractional incorporation values, since unlike specific activity, this measure is independent of the total TdR-3H reaching the tissue. The critical factor is the incorporation of TdR-3H into the DNA from the tissue pool. Once again the more actively proliferating tissues have the higher fractional incorporation values.

Thus the use of F.I. as a measure of DNA synthesis obviates the necessity to attain steady intracellular pool levels of TdR-3H. Nor is it necessary to attain the minimal TdR-3H pool levels to ensure a linear uptake of label into DNA [22]. It also has the effect of ensuring independence from the amount of tritium reaching the tissues and therefore allows for variation in blood flow to large solid tumours, as well as subsequent absorption variations. The need for accurate injection volumes (on a mg/kg basis) is reduced and the effect of environmental variation, which may affect the distribution of this label, is nullified.

Application of F.I. to tissue proliferation

The following data relate the responses of

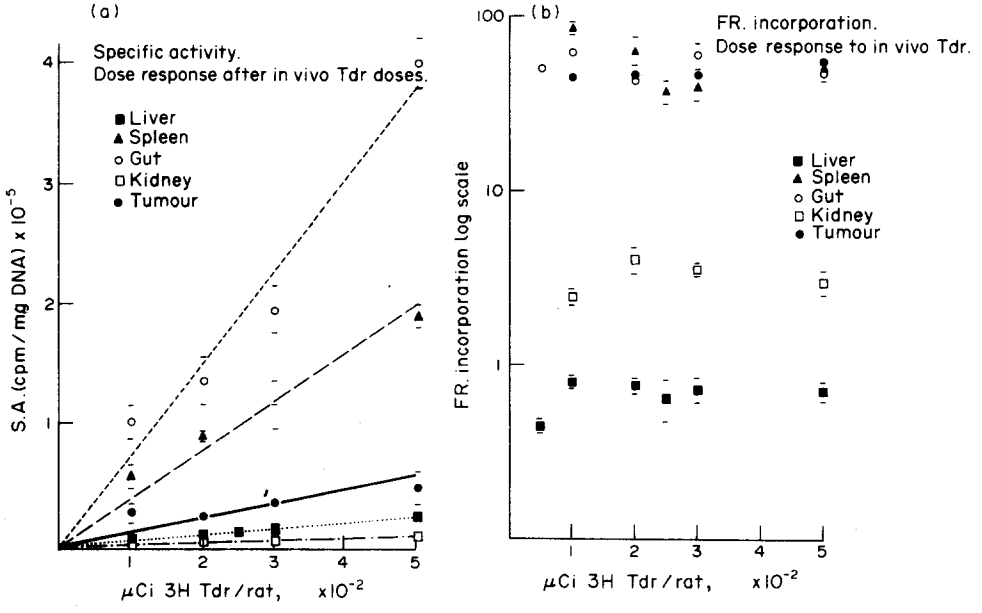


Fig. 4. A comparison of fractional incorporation and specific activity at variable thymidine-6-3H doses. The abscissae are the doses of thymidine-6-3H expressed as μCi/*rat* $\times 10^{-2}$. *Each point is the mean of* 5 *experiments* ±*S.E.*

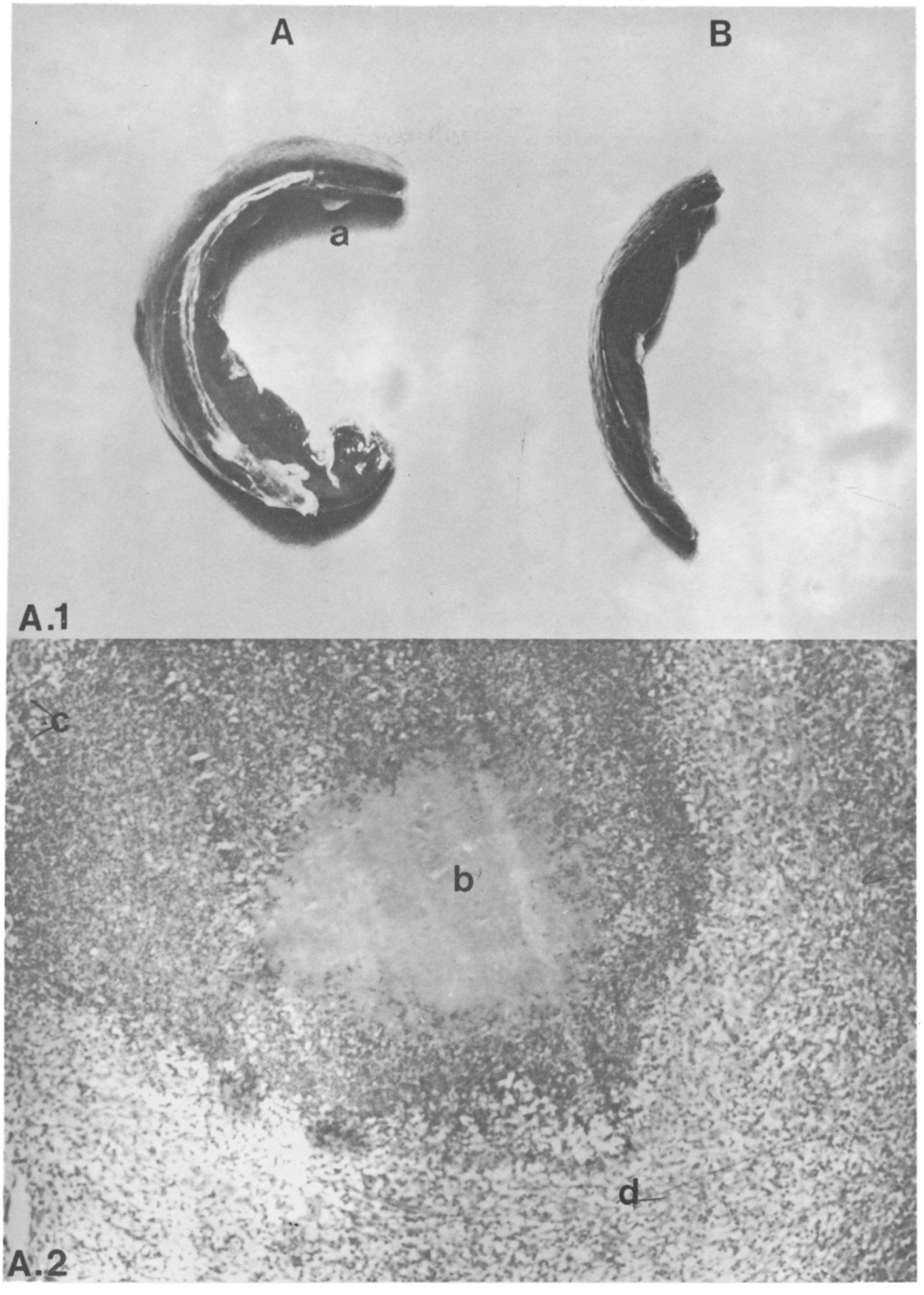
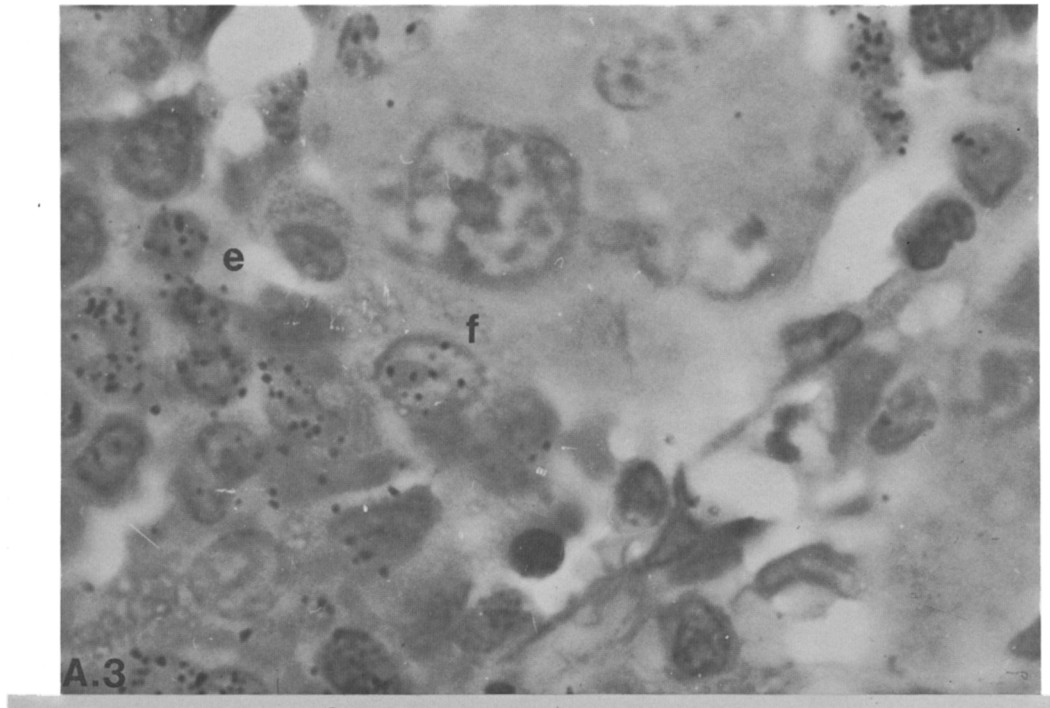


Fig. 5. (*Magnification* ×5) *Comparison of normal spleen from a tumour bearing rat* (*B*) *and an "activated spleen"* 10 days *after a dose of* 100 mg/kg *CY* (*A*). *a* = *pyorrhoeic inflammation.*

Fig. 6. (×40) *Cross section* (*c.s*) *through inflammatory focus* (*a*) *of CY treated spleen, b* = *cell debris and mucus; c* = *white pulp region; d* = *sinusoid red pulp region.*



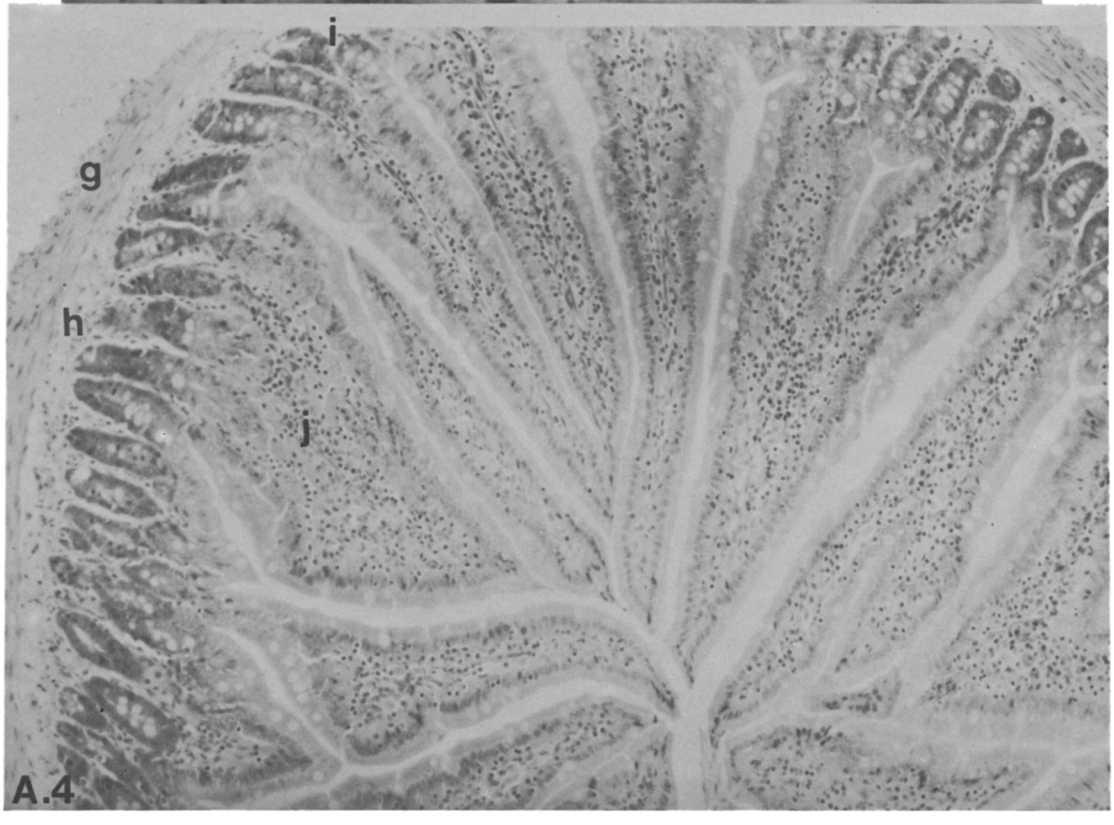


Fig. 7. (×1000) *C.S. high power view of red pulp region* (*d*) *showing tritium grains. The lymphocytes* (*e.g. e*) *and macrophage* (*e.g. f*) *elements appear to be heavily labelled with TdR-3H grains. e = lymphocyte; f = histocyte with granular cytoplasm; nucleus and nucleolus.*

Fig. 8. (×40) *C.S. untreated rat jejunum. g = muscularis mucosa; h = submucosa; i = crypts of Lieberkuhn; j = villi.*

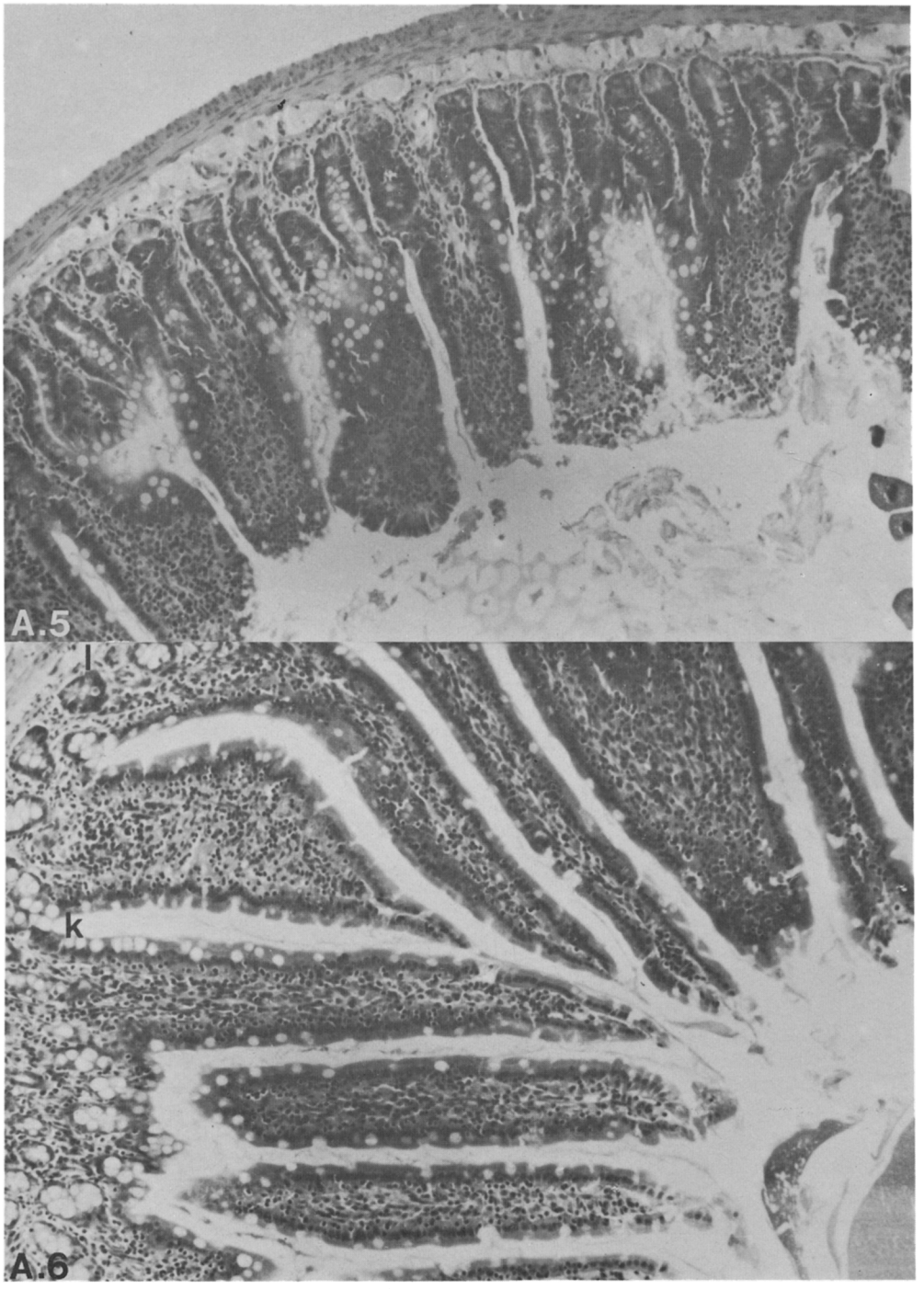


Fig. 9. (×40) *C.S. rat jejunum* 6 hr *after* 5 mg/kg *MTX.*

Fig. 10. (×40) *C.S. rat jejunum* 24 hr *after* 5 mg/kg *MTX. k = beginnings of crypt vacuolisation; l = normal crypt.*

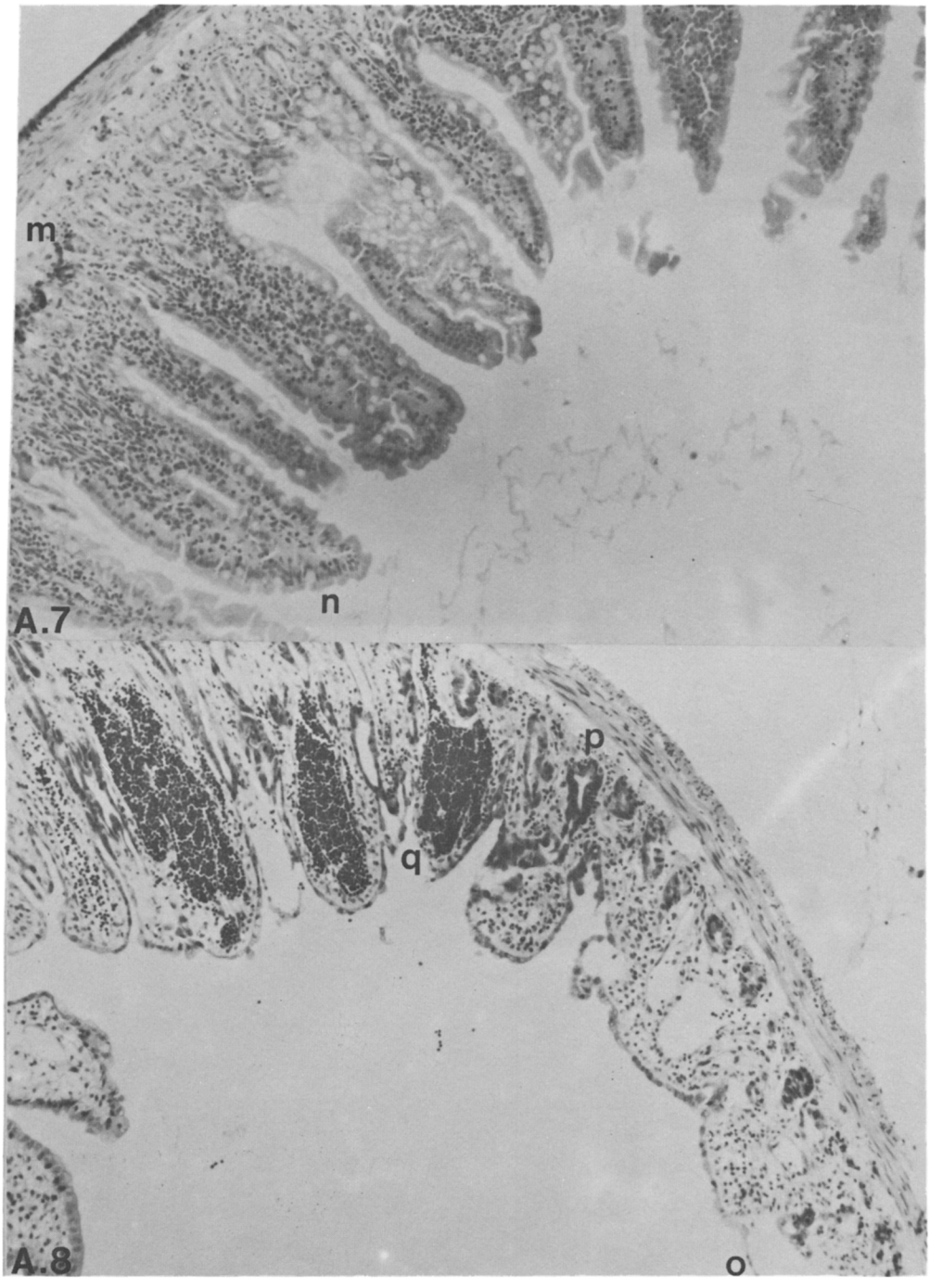


Fig. 11. (×40) *C.S. rat jejunum* 48 hr *after* 5 mg/kg *MTX. m = attritic crypt structure with diffusion of connective tissue; n = abnormal, pitted epithelium.*

Fig. 12. (×40) *C.S. rat jejunum* 72 hr *after* 5 mg/kg *MTX. o = grossly abnormal villus epithelium: p = possible sites of crypt cell regeneration: q = possible infection sites—lymphocytic activity.*

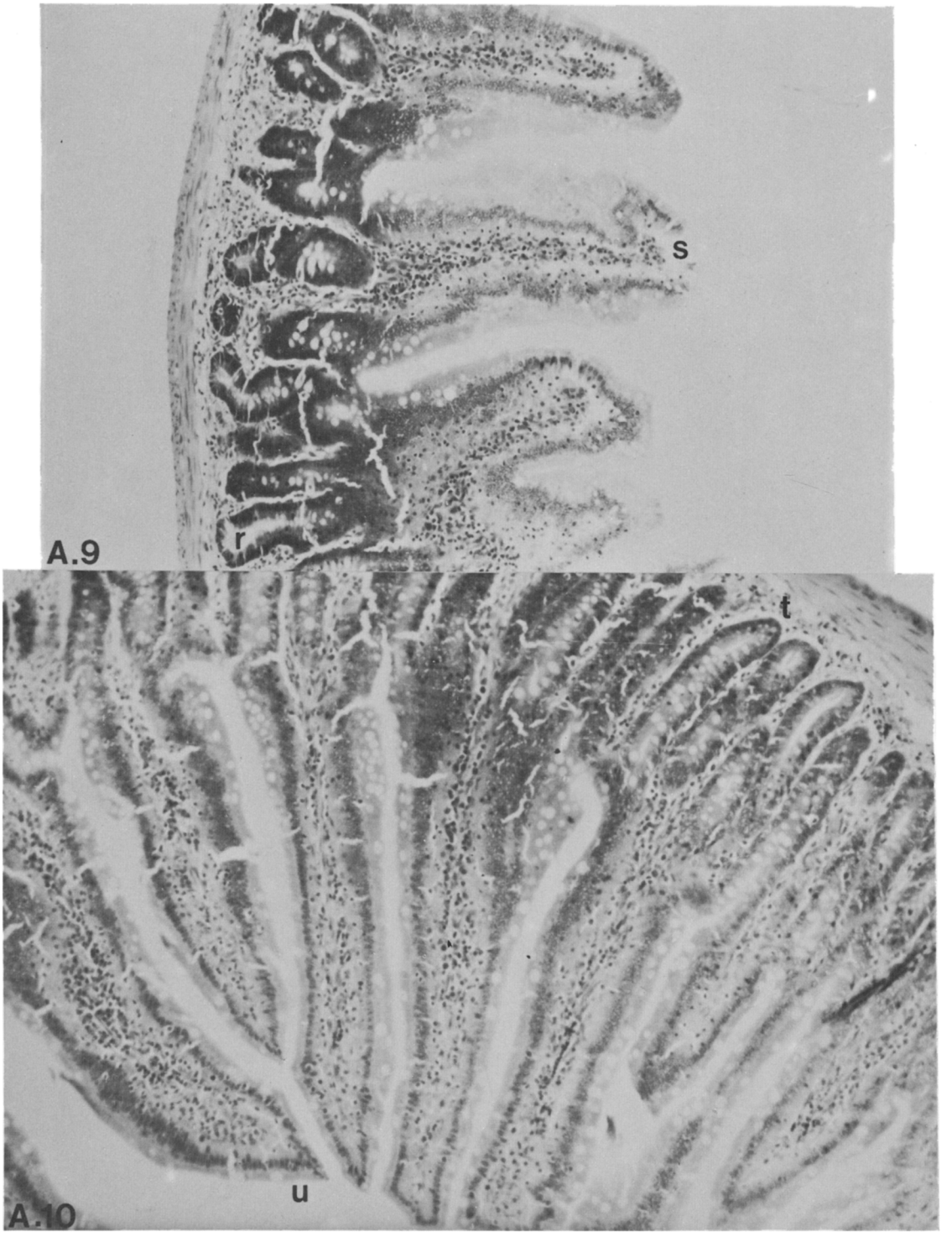


Fig. 13. (×40) *C.S. rat jejunum* 144 hr *after* 5 mg/kg *MTX. r = crypt hypertrophy* (2–3 *cell layers*) *and gross lymphocyte involvement; s = healthier villus structure.*

Fig. 14. (×40) *C.S. rat jejunum* 170 hr *after* 5 mg/kg *MTX. t = crypts still slightly hypertrophic and elongated; u = villus structure and epithelium healthy.*

Table 2. *The effect of* 100 mg/kg *CY on spleen proliferation*

Hr After CY	Weight (g)	DNA content (μg/mg wet wt)	F.I. TdR-3H
Tumour-bearing control	429	7.5	82
24	312	2.7	1.3
48	204	2.5	4.0
72	241	2.4	7.2
120	359	2.0	32
164	262	2.7	68
240	927	11.3	98.9

Values are mean of at least 5 experiments.
Standard errors $< 10\%$ mean.

spleen and intestinal mucosa (jejunal) to treatment with CY and MTX respectively and attempt to correlate F.I. measurements with non-biochemical parameters of tissue proliferation. Correlation of F.I. with tumour volume response [5, 7] and bone marrow cellularity [23] after cytotoxic drug treatment have been illustrated previously.

Spleen

The effect of a single dose of 100 mg/kg CY (LD5) upon spleen weight, DNA content and DNA synthesis is shown in Table 2. Table 2 shows that the CY caused a decrease in all three parameters within the first 24 hr, recovery ensued between 7 and 10 days. The weight and volume (Fig. 5) were double the pre-treatment value by 10 days. The DNA content was 50% increased. The F.I. value was much increased, the 98.9 value signifying that all but 1% of the TdR reaching the spleen was incorporated into DNA. The total levels of 3H reaching the spleen were also significantly increased as compared with control.

Figure 5 illustrates the difference in volume between a control and treated spleen. As well as the difference in size, white nodules were present in the treated spleen. These were pyorrhoeic inflammations containing dead cells and mucus (Fig. 6). Whilst the increase in size after treatment was highly reproducible, the formation of these inflammatory foci was a rare occurrence. An autoradiograph of the red pulp region of the treated spleen is also shown. The lymphocyte and macrophage populations incorporated high levels of TdR-3H so presumably these cells were the most actively dividing and would account, at least in part, for the observed increase in F.I. Consideration of spleen function would suggest that the increased splenic activity after treatment was caused by a "rebound-effect" either as a direct result of drug treatment or indirectly due to infection. Since a similar effect was found using methotrexate [5] this spleen reaction was not a specific CY mediated response.

Intestinal mucosa

Table 3 summarises the effect of 5 mg/kg MTX on parameters of jejunal cell proliferation. Evidence of lymphocyte involvement (as well as crypt and villus structure) was gained by studying stained cross sections of jejunum. Representative examples of these sections are shown in Figs. 7–14.

DNA synthesis measurements are complicated by the balance of supply of thymidylate from salvage and *de novo* sources. Methotrexate restricts the supply of *de novo* thymidylate (measured by 3H-UdR) and the concomitant toxicity to the intestinal mucosa results in reduction in 3H-TdR incorporation. The F.I. value for 3H-TdR had recovered between 72 and 120 hr after MTX treatment and although 3H-UdR took longer to recover it is likely that the 3H-TdR incorporation is a better parameter for judging tissue recovery, since it is independent of MTX induced changes in endogenous dUMP pools [24]. The depression and recovery pattern of the 3H-TdR F.I. data was mirrored by animal weight and mucosal DNA content. Maximal DNA loss occurred at 24 hr and this was followed by a period of recovery. DNA synthesis began to recover between 48 and 72 hr and this corresponded with microscopic recovery since some regeneration was visible by 72 hr. The crypt hypertrophy observed at 144 hr was not reflected in an overshoot in F.I. values. This was probably because the total number of crypts was less than control. The smaller number were longer and contained two to three layers of dividing cells, as opposed to the control thickness

Table 3. The effect of 5 mg/kg MTX on parameters of jejunal cell proliferation

Hours after CY	% Pre-treatment body wt	Mucosa† DNA content μg/mg wet wt	3H-TdR† F.I.	3H-UdR† F.I.	Evidence of gut haemorrhage	Lymphocyte involvement		Crypt structure	Villus structure
Control	100	4.6±0.3	76.7±5	41	−	−	8*	Normal	Normal
6	99.6	4.0±0.2	ND	ND	−	−	9	Some cell-death	Slightly shortened
12	94	1.2±0.1	7.1±0.3	6.2±1.2	++	ND		ND	ND
24	90	1.1±0.2	31.0±16	4.8±0.4	++	+	10	Vacuolisation	Epithelial fraying
48	84	2.0±0.5	12.9±5	5.0±0.6	+	+	11	Attritic, invaded by diffuse connective tissue	Shortened, epithelium pitted and irregular
72	85	2.2±0.6	38.2±7	7.7±1.9	+	++	12	Some regeneration, size abnormal. Still with diffuse connective tissue	Grossly shortened, epithelium thin and and broken
120	89	3.3±1.0	68.8±6	19.9±2	−	ND		ND	ND
144	90	3.3±0.8	73.5±12	ND	−	++	13	Hypertrophic Regeneration, Crypts enlarged.	Regaining normal internal structure. Epithelium more complete
170	95	4.1±0.5	61.3±10	38.1+4.6	−	+	14	Still slightly elongated. Divisions normal	Normal

ND = Not done.
*Numbers represent corresponding photomicrographs.
†Mean of 4 experiments ± S.E.

of one cell layer. In general there was good correlation between the F.I. values and the morphological appearance of the jejunum.

The gut recovery data may assist in providing an explanation for the findings of Harding *et al.* [25] who showed that the body weight of their rats was depressed if a second dose of MTX was administered 2–6 days after a first, but not if given later. Our temporal scheduling method for two alkylating agents [23] makes use of the recovery of F.I. in host target tissues. We have shown that the most animal-sparing combination occurs when these target tissues are allowed to recover before administration of a second insult. Thus, in the above example, F.I. in the jejunum has recovered by days 7–8 and administration of a second MTX dose at this time should cause minimal gut toxicity.

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BCG-Immunotherapy of L_2C-Guinea Pig Leukemia

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Abstract—*Guinea pigs of inbred strain 2 were treated by an intradermal injection with a lethal dose of* 10^5 *gelatine purified leukemic blood leukocytes in mixture with* 10^7 *colony-forming units (CFU) of BCG strain Copenhagen* 1331. *Of* 56 *animals inoculated in this manner* 66% *showed a significantly* ($P=0.05$) *improved life expectation in comparison to* 48 *guinea pigs not BCG-treated, all perishing of leukemia within an average of* 22.4 days (±3.9 *S.D.*) *after tumor cell application. BCG enabled* 19% *of the animals to survive* L_2C*-inoculation more than* 15 *weeks. Eighteen of these survivors were challenged with* 10^5 L_2C*-leukocytes,* 15 *of them remained healthy demonstrating an acquired resistance against this leukemia. Positive tuberculin reaction converted to negative in the three susceptible, challenged animals and was transiently missing in two resistant guinea pigs with a spontaneous remission of histologically apparent leukemia. BCG was useless when injected separately from* L_2C*-leukocytes or brought into contact with tumor cells* 15 days *after implantation. Antitumor activity of* L_2C*-cells adjoining BCG was furthermore hindered by BCG-vaccination six to seven weeks before immunotherapy. Increase of bacterial mass from* 1×10^7 *to* 2×10^8 *CFU for immunotherapy did not restore the antileukemic effect. BCG-induced ulceration at the site of injection of* L_2C*-leukocytes occurred in pre-sensitized animals much earlier than in unvaccinated guinea pigs. These results are discussed with respect to the presumably restricted possibilities of BCG acting against leukemia in animal models.*

INTRODUCTION

THE EFFICACY of immunotherapy with Bacillus Calmette-Guérin (BCG) to prolong the chemotherapy induced remission in leukemia patients is not definitively clarified yet. Early results from the BCG-treatment of acute myelocytic leukemia (AML) were hopeful, [1–4] but not conclusive because inappropriate control groups had been chosen. The same restrictions must be noted for the studies concerning the immunotherapy of chronic myeloid leukemia (CML) [4, 5]. Encouraging work has been done to prolong the remission of patients with acute lymphoblastic leukemia (ALL) by BCG [4, 6] but follow-up studies have failed to confirm these results. [7, 8].

All these clinical trials were based exclusively on findings derived from different experimental murine leukemias as the Rauscher leukemia [9], the Gross leukemias E♀K1 [9] and E♂G2 [10], the Moloney leukemia LSTRA [11, 12], the L1210 leukemia [13, 14] and especially the spontaneous leukemia of the AKR-mouse [15–17]. To obtain additional information we investigated a new model in BCG-research, the L_2C-guinea pig leukemia.

This leukemia, having spontaneously appeared in 1954, was isolated from an animal of inbred strain 2 and transmitted further by serial transplantations [18]. The advantages of the model are: firstly, unlike many chemically induced tumors, the guinea pig leukemia is weakly immunogenic [19]; secondly, and more importantly, cellular immunity appears to be the predominant, if not exclusive, factor in immunoprotection against this disease [20]. This resembles the situation in human leukemias where cellular immunity appears to be of decisive importance against ALL [21–24], AML [21, 22, 24, 25] and CML [5, 26]. As opposed to the mouse, the guinea pig exhibits a tuberculin reaction [27, 28] nearly identical to that in man [29, 30]. This is a further advantage of the L_2C-system because the tuberculin reaction reflects changes in cellular immunity in leukemic patients very simply [5, 21, 23]. In addition, the L_2C-leukemia is attractive because of its similarity to the ALL of childhood with regard to pathology [31] and to the meningeal form after chemotherapy [32]. Sensitivity to this leukemia is not age dependent [33] and the thymus of leukemic animals is not

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affected [33, 34]. Terminal stages of the illness show a severe thrombocytopenia [35]. With respect to these characteristics the L_2C-leukemia may prove to be the most valuable model for the study of ALL [33].

In the same inbred strain of guinea pigs a hepatocarcinoma was described. It was often used as a model for BCG-imuunotherapy [36–40], and therefore it was possible to compare susceptibilities of a leukemia and a solid tumor to BCG in a genetically identical environment.

The following experiments reveal some features concerning the sensitivity of the L_2C-leukemia to BCG-treatment and the effect of BCG-vaccination prior to immunotherapy. Subsequently, we will discuss the differences which possibly exist between solid and leukemic neoplasias to enforce optimum anti-tumor efficacy of BCG.

MATERIAL AND METHODS

Guinea pigs of inbred strain 2 [41] were obtained directly from Altick Ass. Hudson (Wisc. 54016) by air carriage. Inbred status of the animals was checked by skin transplantation between three pairs of males selected from different litters. The technique employed has been described by others in detail [42], but we anaesthetized by inhalation of Penthrane (Deutsche Abbott GmbH, Ingelheim). We kept the guinea pigs free of endoparasites in groups of three to four in stainless steel grid cages at room temperatures of 295K with a relative air humidity of 55%. Animals were fed with dry pellets of guinea pig chow (Intermast, Soest, Ger.) and water continuously available. They got carrots twice a week. Only males were used for BCG experiments.

L_2C-leukemia cells were obtained from D. Gericke (Hoechst AG, Ger.), who got the material in an unknown passage generation from L. Gross. Leukemic spleen cell preparations in Hanks' solution, pH 7.0, with 10% DMSO were frozen and stored in liquid nitrogen in the usual manner [43]. Sterility controls of cell suspension for freezing or for experiments were done with samples in thioglycolate medium at 293 and 310K, in Sabouraud medium at 293K and on blood agar plates at 310K. Only in the first experiment did we use these frozen spleen cells as leukemic inoculum. It turned out to be too risky to use fresh cells as there was frequent contaminations of the spleen during preparation. For this technical reason all other experiments were begun with a fresh leukocyte preparation.

Citrated blood was taken from an anaesthetized terminal leukemic animal by cardiac puncture and leukocytes were purified in gelatine [44]. We centrifuged the leukocyte suspension at $200 \times g$ for 20 min and then after washing once more, for 10 min. Cells were counted and viability assessed with use of trypan blue exclusion. The proportion of undifferentiated blast cells did not change markedly during purification procedure as estimated by Pappenheim staining. Samples were separated just before use to test sterility as described above. Leukemic leukocytes were applied in disposable plastic syringes. White blood cells of L_2C-inoculated animals were estimated three times a week by using a cell-counter (TOA Medical Electronics, Kobe, Jap.). Measurement of the hemoglobin content was done optically with a Hb-meter (American Optical Corporation, Buffalo).

Frozen BCG (Strain Copenhagen 1331) was employed only in the first experiment using leukemic spleen cells as tumor inoculum. The bacteria were harvested from a dispersed culture in Sauton-medium with 0.2% tween at 310K. The yield was washed, resuspended in stabilizer and stored at 243K. Before use, BCG was thawed quickly and an equal volume of *aqua bidest.* was added. *Fresh BCG* of the same strain was used in the second experiment in mixture with L_2C-leukocytes. These bacteria were grown in a dispersed culture as described before but used immediately after harvesting. *Lyophilized BCG*, a commercial product of the Behringwerke AG (Marburg, Ger.), was used in the majority of the experiments. To prepare this vaccine (K.-Nr. 240-1) BCG of strain Copenhagen 1331 was cultured for 7 days in Sauton-medium at 310K. Subsequently, pellicle grown bacterial mass was grounded with steel balls, centrifuged and resuspended in a stabilizer fluid consisting of 3.5% Haemaccel® (registered trade mark of Hoechst AG), a degraded gelatine and 5% glucose before lyophilization. *Colony forming units* (CFU) were estimated by spreading appropriate dilutions of the BCG-suspension on Dubos-agar plates enriched with 5% horse blood. Colonies were counted after 4 weeks of incubation at 310K. *Dry weights* were determined by using 0.45 μm filters (Sartorius, Göttingen, Germany) 50 mm dia and dried at 378K for 24 hr before and after loading with bacteria. Dilutions of BCG were done in stabilizer after addition of an equal volume of *aqua bidest* to keep an isotonic environment.

Immunotherapy was done for the most part by adding BCG to a lethal dose of L_2C-material and injecting the mixture intradermally. Exact concentration applied, body weights of guinea

pigs and modifications of the experimental procedure are described in detail in figure legends. All control groups without BCG were inoculated at the end of each test to exclude a possible loss of activity during injection procedure.

Tuberculin tests were carried out with old tuberculin (Behringwerke AG, Marburg) containing 100,000 i.u./ml. After a dilution of 1:100 with physiological NaCl, 0.1 ml of this solution was injected intradermally in the shaved dorsal region of the guinea pig. Largest diameter of induration, not erythema, was measured 24 hr later; a diameter of 5 mm or more was accepted as a positive skin reaction [28]. All injections and readings were done by the same person.

Statistical analysis of survival times of guinea pigs in different groups after implantation of leukemic material with or without BCG was adapted to the observation that in BCG-treated groups some animals survived, and therefore the distribution of life times possesses an open flank, which was never observed in control groups, where animals died without any exception. Thus, we calculated the distribution of life duration with its different probabilities only for control groups according to Student's *t*-values. The number of animals with life prolongation in a BCG-treated group was expressed as the percentage beyond the upper confidence limit of $P=0.05$ in the corresponding control group. In the experiments 6 and 7 we compared animals life data in the two pre-sensitized groups using the one-tailed test of Mann and Whitney.

RESULTS

One of the limitations of a successful immunotherapy in animals as well as in man is a high number of tumor cells in the organism. In murine systems for example the critical concentrations are 10^4 for the E♂G2 [10] and 10^5 for the L1210 leukemia [13]. More than 10^5 leukemic cells may overwhelm the possible benefit of BCG in the guinea pig system too. On the other hand, application of a reduced cell number would allow some guinea pigs to survive without support of BCG because 10^5 L_2C-leukocytes injected intradermally are necessary to cause 100% deaths [20]. For all the following experiments we therefore used a transplantation dose of 10^5 tumor cells.

Experiment 1: *BCG in mixture with L_2C-spleen cells*

In the first experiment spleen cells were used as tumor inoculum. One group of guinea pigs received leukemic cells alone, the other one got the same dose in combination with BCG. Details of the procedure are exemplified in the legend to Fig. 1. To consider possible toxic effects of BCG suspending medium against spleen cells, the amount of viable L_2C-cells was determined after finishing all injections, but a distinct decrease was not observable. About one week after inoculation all animals developed a small intradermal nodule at the side of L_2C-injection. The BCG-group was

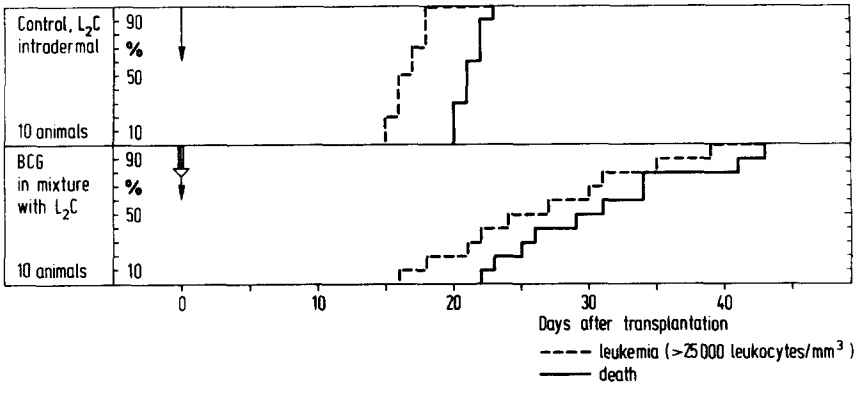

Fig. 1. Cumulative mortality of guinea pigs inoculated with a mixture of L_2C-spleen cells and BCG.

A frozen L_2C-spleen cell suspension from a female with 224,000 *leukocytes/*mm^3 *and an amount of* 91% *leukemic blast cells in the peripheral blood was thawed rapidly in a* 310*K water bath. Each guinea pig of the test group got intradermally* 0.5 ml *BCG suspension (broad arrow) containing* 1.4×10^6 *CFU (determined after thawing) and a dry weight of* 0.032g *in mixture with* 0.5 ml *of a* 1:100 *dilution of the spleen cell suspension. Control animals were inoculated with* 0.5 ml *spleen cells in mixture with* 0.5 ml *stabilizer* 1:2 *diluted with* aqua dest. *Each animal got* 1.3×10^5 *viable and* 3.0×10^4 *dead spleen cells or a better ratio of this (thin arrows). Median animal weights were* 581g *for the test and* 580g *for the control group at the beginning of the experiment.*

totally tuberculin positive on day 9. In this skin test the other group, which had received spleen cells alone but remained healthy during the test, served as a negative control. Beginning with day 20 an inflammatory reaction characterized by induration, ulceration and necrosis developed in the BCG group in all animals but with individually different degrees. As shown in Fig. 1 all guinea pigs subsequently succumbed to leukemia. The onset of disease was however considerably delayed in the BCG-group. Herein 80% of the animals have a significant ($P=0.05$) extension of life span although 0.05% DMSO present in the spleen cell suspension at the time of injection might hinder activity of BCG.

Experiment 2: *BCG added to L_2C-leukocytes at different times*

In the next experiment fresh leukemic leukocytes from the blood were purified. Using the procedure described in Material and Methods only viable cells were obtained. Results of this BCG-experiment are presented in Fig. 2. The first group of guinea pigs which served as a control received only the tumor cells. These animals developed moderate, intradermal nodules. The onset of leukemia and occurrence of death took place in the same period after transplantation as observed for the control group in experiment 1. The second group of guinea pigs got L_2C-leukocytes and BCG in mixture. Ulcerations were observed two weeks after transplantation. An antitumor activity of BCG was noted in this group by a significant ($P=0.05$) life-prolongation of seven animals. Of these, two guinea pigs were still alive 15 weeks after tumor implantation. And these will be referred to later as long-term survivors. Animals of the third group were inoculated with tumor material at the beginning of the experiment and received BCG 15 days later by injection into intradermal tumor nodules. As can be seen in the graph, two animals of this group were leukemic at this time, but neither these guinea pigs nor the rest of the group showed an improvement of life-expectation or duration of disease by BCG-treatment although ulceration of the nodules were noticed. Therefore, time of BCG-employment and not ulceration of tumor nodules was crucial in antileukemic activity of BCG.

Experiment 3: *measurement of BCG-antitumor activity in the L_2C-system*

In the preceding experiment the number

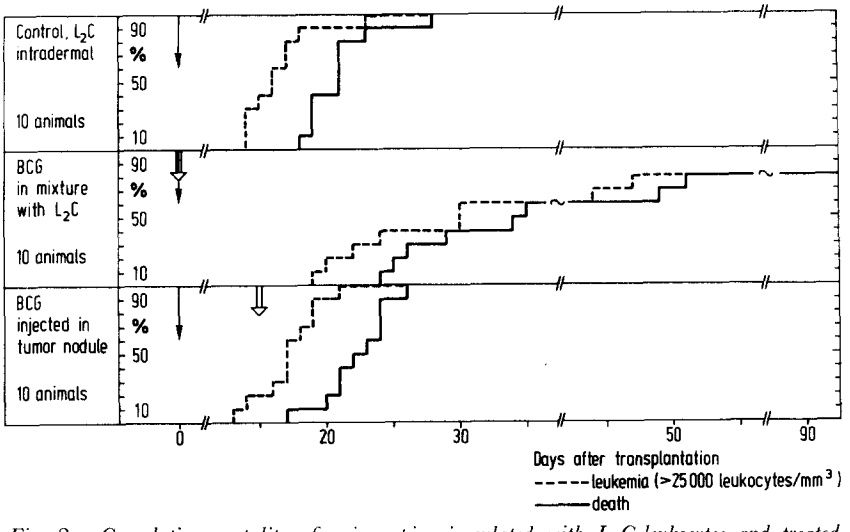

Fig. 2. Cumulative mortality of guinea pigs inoculated with L_2C-leukocytes and treated with BCG at different times.

Leukocytes were prepared from a male with a white cell blood count of 310,000/mm^3 *and a portion of* 60% *blast cells in peripheral blood. Each animal got* 0.5 ml 1.1×10^5 *viable tumor cells intradermally (thin arrows). Guinea pigs of the second group received in mixture with these leukocytes* 0.5 ml *BCG with* 7.8×10^7 *CFU and* 1.3 mg *dry weight (broad arrow in the middle panel). Despite washing BCG twice with physiological* NaCl, *traces of detergent derived from culture medium might have been present and toxic for leukocytes. To exclude this possibility,* 0.5 ml *of the* 0.45 μm *filtered (Millex, Millipore) first BCG-washing were added to the leukocytes of the control group. When tumors were palpable at the injection site in all animals of the third group,* 0.2 ml *BCG with* 5.2×10^6 *CFU and* 0.07 mg *dry weight from a fresh liquid culture was applied locally (broad arrow in the lowest panel). Median animal weights were* 488g *in the first,* 489g *in the second and* 490g *in the third group as estimated just before start.*

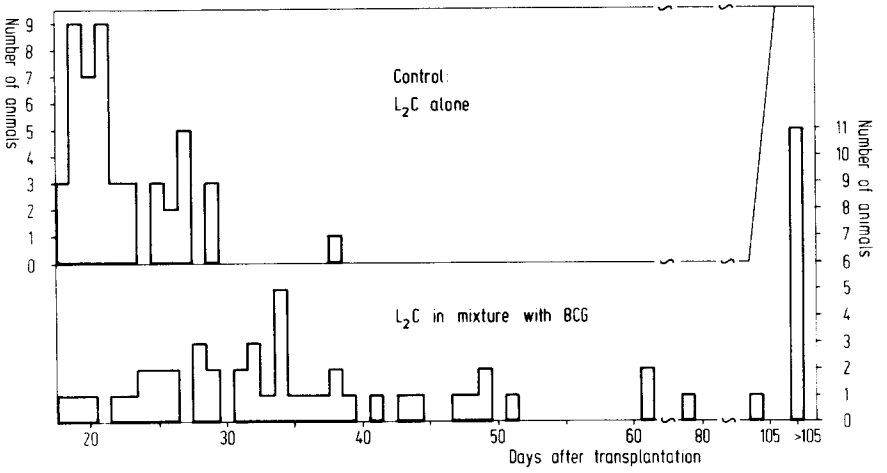

Fig. 3. Life time-histogram of guinea pigs after intradermal inoculation of L_2C-leukocytes. In the immunotherapy group each animal received 0.5 ml *L_2C-leukocytes with about* 1.0×10^5 *cells in mixture with* 0.5 ml *BCG strain Copenhagen containing nearly* 1.0×10^7 *CFU. Control guinea pigs got* 0.5 ml *of the same leukemic material after addition of* 0.5 ml 1:2 *diluted stabilizer to obtain an environment for leukocytes comparable to that in the immunotherapy group.*

of guinea pigs was not sufficient to calculate percentages of animals with BCG-induced life prolongation or ability to survive. For technical reasons it was not possible to enlarge the basis for this calculation in one extensive experiment. Therefore, three unrelated tests were done in an identical manner as experiment 2 but omitting the third group. Combining experiment 2 and the three tests of experiment 3 a total of 56 guinea pigs were inoculated with a mixture of L_2C-leukocytes and BCG; as a control 48 animals were not BCG-treated. As shown in Fig. 3 all control animals died between day 18 and 38 after transplantation with an average of 22.4 ± 3.9 S.D. (standard deviation). As demonstrated in the same graph BCG improved life expectation considerably for many guinea pigs. In comparison to the corresponding controls of each test a total number of 37 BCG-treated animals (66%) possessed a significant ($P = 0.05$) extension of life including 11 animals (19%) which survived L_2C-transplantation for more than fifteen weeks.

Experiment 4: rechallenge of animals surviving by BCG-immunotherapy

Eighteen guinea pigs, which survived L_2C-transplantation in the experiments described before and in an accompanying study comparing the antitumor activities of different BCG-vaccines, were challenged with 10^5 L_2C-leukocytes applied intradermally as described above but now without adding BCG. All these survivors were tuberculin positive as tested some days before the experiment. The challenge was carried out 92–184 days after the first L_2C-inoculation.

Since a positive tuberculin reactivity might influence the course of leukemia by itself, animals of the challenge control group were sensitized with BCG 4–17 weeks before use. These control animals, 33 in total number, all died of leukemia within an average of 25.6 days (± 4.7 S.D.) after L_2C-inoculation.

Reaction types observed in the rechallenged group are summarized in Table 1. Out of

Table 1. Observed reactions of eighteen long-term survivors after rechallenge with L_2C-leukocytes

Reaction types	Number of guinea pigs	Animals with reduced hemoglobin content in the blood	Disappearance of tuberculin reactivity
Leukemia	3	3	Yes
One or two attacks of leukemia with remission	2	1	Transient
L_2C-resistent	13	1	No

Results are explained in detail in the running text.

the 18 survivors challenged only three animals died of leukemia 34, 59 and 74 days after tumor cell application respectively. For all these animals a loss of positive tuberculin reaction was observed accompanying the neoplastic disease.

Forty-four and 90 days after beginning of the challenge another guinea pig showed elevated leukocyte concentrations in the peripheral blood up to 34,000 cells/mm^3, 1–2% in typical leukemic forms. Both events were in parallel with a transient conversion from tuberculin positive to negative. Very surprisingly, this leukemic animal recuperated each time in parallel with a slow restoration of the capability to react in the skin test. A self limiting disease was observed in a second animal too, in which 121 days after start of challenge leukocytes increased to 33,000/mm^3 with typical blast cells in the peripheral blood and a simultaneous loss of delayed hypersensitivity against tuberculin. As in the case described above, a spontaneous remission of leukemia occurred for some days accompanied by a restoration of tuberculin reactivity.

The majority of the challenge group, thirteen animals, remained continously healthy for several months demonstrating a strong acquired resistance against L_2C-leukemia. These guinea pigs were tuberculin positive during challenge and served as a positive control for the skin tests with negative results as mentioned above.

Two challenged animals, one of the L_2C-resistant and one of the guinea pigs with the single spontaneous remission, exhibited a decline of erythrocytes concentration to 2.9×10^6/mm^3 and 3.3×10^6/mm^3 three months after challenge. At this time erythrocytes concentrations tested in other animals of the challenge group were normal with a mean value of 5.5×10^6/mm^3, very similar to that of untreated guinea pigs of inbred strain 2. Against a normal content of 15g hemoglobin per 100 ml, blood concentration of this substance was strongly reduced in both animals to 10g and 8.5 g/100 ml respectively, a level normally measured only in leukemic guinea pigs. Additionally, these animals showed loss of body weight. Illness, presumably of the hematopoetic system, disappeared very slowly after some weeks.

Experiment 5: *BCG-vaccination before BCG-immunotherapy*

Since a positive tuberculin reaction is very common in humans [30], we investigated in the following experiments the influence of this immunologic state on antitumor activity of BCG in the L_2C-system. Guinea pigs were sensitized by intradermal injections with BCG. Six weeks thereafter animals received the usual dose of L_2C-leukocytes plus BCG. One control

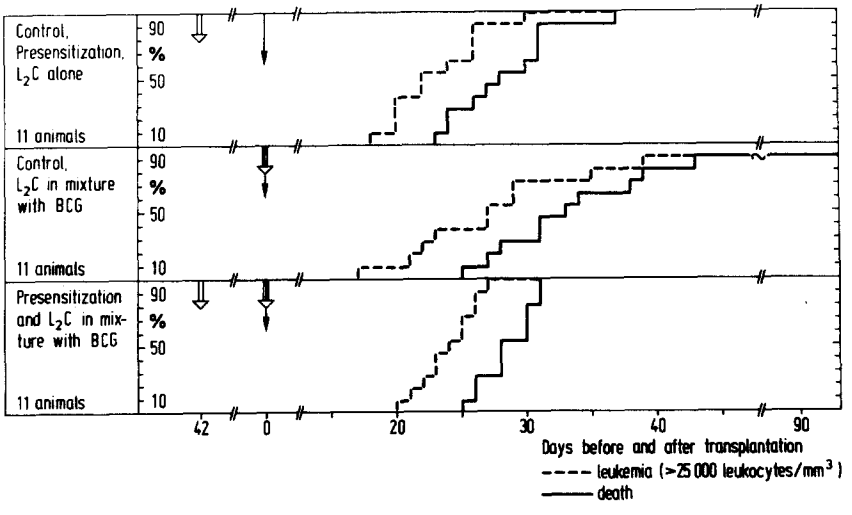

Fig. 4. Cumulative mortality of guinea pigs inoculated with a mixture of L_2C-leukocytes and BCG. Effect of intradermal pre-sensitization with BCG.

Animals of two groups were sensitized by injecting 0.1 ml *with* 4.3×10^6 *CFU BCG in each flank intradermally (broad arrows on day* 42 *before transplantation). A guinea pig with a leukocyte count of* 177,000/mm^3 *and a portion of* 84% *leukemic cells in the peripheral blood was sacrificed for leukocyte preparation. From this, all animals in the experiment received* 0.5 ml *with* 0.8×10^5 *L_2C-cells (thin arrows), two groups in mixture with BCG containing* 1.0×10^7 *CFU and* 0.065 mg *dry weight (broad arrows on day* 0), *one group with* 0.5 ml 1:2 *diluted stabilizer instead of BCG (upper panel). Median animals weights were* 574g *for the first,* 599g *for the second control and* 587g *for the actual test group.*

group got this mixture without pre-sensitization in a manner identical to the tests mentioned in experiment 3, a second control group was vaccinated with BCG, but inoculated with L_2C-leukocytes alone. As shown in Fig. 4 in this experiment pre-sensitization with BCG abolished the antitumor activity of BCG totally, because both BCG-vaccinated groups have exactly the same mean of death-time, that is within 28.4 days after L_2C-inoculation regardless of adding BCG to leukemic material or not. Antitumor activity of BCG was reduced by pre-sensitization to such an extent that this group resembles the usual negative control groups receiving L_2C-cells alone. It was not possible to decide if BCG-vaccination itself has a weak benefit against L_2C-leukemia because a suitable control was missing here.

Experiment 6: *BCG in L_2C-prophylaxis and BCG injected simultaneously but separately to the tumor cells*

Since a possible prophylatic effect of BCG-vaccination against human leukemias is now under discussion [45] a comparable situation was tested in the L_2C-system. Hereto the first group of guinea pigs was vaccinated with BCG and inoculated with L_2C-leukocytes seven weeks later. Another question to be answered was the possible requirement of contact between BCG and tumor cells for successful immunotherapy. Therefore animals of the second group received BCG and L_2C-leukocytes by contralateral intradermal injection. In addition, to confirm the results of experiment, 5 guinea pigs of the third group were pre-sensitized with BCG and treated with the BCG–L_2C-mixture later. The fourth and fifth group which consisted of non-vaccinated animals received L_2C alone as a negative control or submitted to usual immunotherapy as a positive control. Results of the experiment are depicted in Fig. 5. Since in our experiments an extension of leukemia, that means the duration of the neoplastic disease, by BCG was never observed, only the lines for cumulative death are entered in Fig. 5. Nevertheless, each animal enumerated died of leukemia.

Three main types of reaction were observed for the five different groups. The group receiving BCG and L_2C-material at different sites was not statistically distinguished from the animals inoculated with only L_2C-leukocytes. This was also observed in the case of BCG-vaccinated animals inoculated with the L_2C-cells some weeks later. But here there was a remarkable exception: one animal survived 102 days after L_2C-implantation. Since this guinea pig was tuberculin positive after vaccination, it might be possible that in rare cases BCG-vaccination alone has an effect against L_2C-leukemia.

Another type of reaction in the positive control group was the antitumor activity of BCG against accompanying tumor material as expressed in animals with improved life expectation and the occurrence of survivors. This was expected for successful immunotherapy.

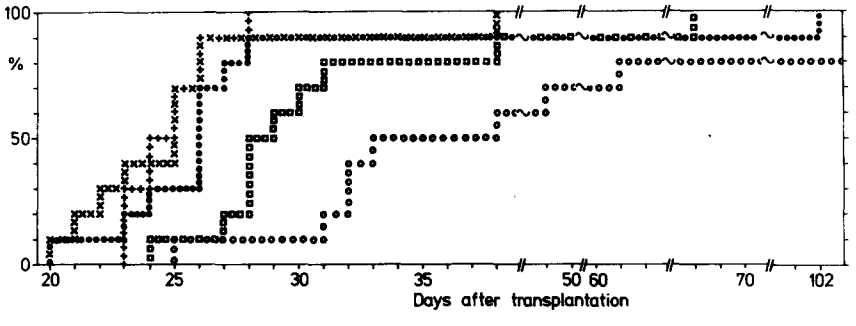

Fig. 5. Cumulative mortality of guinea pigs inoculated intradermally with L_2C-leukocytes and treated with BCG in different ways.
×—*L_2C alone;* ●—*sensitized with BCG* (−49 days), *L_2C alone;* □—*sensitized with BCG* (−49 days), *L_2C in mixture with BCG;* ○—*L_2C in mixture with BCG:* +—*L_2C and BCG at different sites.*

Each group consisted of 10 *animals. Guinea pigs of two groups were sensitized by injecting* 0.1 ml *with* 7.3×10^5 *CFU of BCG intradermally in each flank* 49 days *before starting transplantation. For this, leukemic material was prepared from an animal with* 290,000 *leukocytes*/mm^3 *and a portion of* 88% *blast cells in the peripheral blood. On day* 0 *all animals received* 1 ml *of* 0.5 ml *with* 1.2×10^5 *L_2C-leukocytes supplemented with* 1.0×10^7 *CFU and* 0.065 mg *dry weight of BCG or with* 1:2 *diluted stabilizer. Animals of one group got leukocytes and BCG separately, but both in doses equal to those applied in the other groups. Median animals weights ranged from* 589 *to* 622 g *for the five different groups.*

The last type of reaction was intermediate between the two described above and was measured for the pre-sensitized group inoculated with a mixture of L_2C-cells and BCG. A slight life prolongation of some guinea pigs was recognizable, but survivors were absent. Pre-sensitization with BCG reduced antitumor activity of this *Mycobacteria* applied later, but not to the same extent as observed in the foregoing experiment. This becomes clear by calculating the differences between both pre-sensitized groups; mixing BCG with leukemic material improved life expectation ($P=0.01$) in opposition to the experiment outlined in Fig. 4.

To investigate the state of cellular immunity in this experiment delayed hypersensitivity was measured repeatedly. Guinea pigs pre-sensitized with BCG were checked four days before L_2C-implantation by tuberculin test and found to be all positive. Here some animals from the groups not BCG-vaccinated served as a negative control. Ten days after L_2C-inoculation the tuberculin test was repeated. All animals were tested except those which had received BCG and L_2C separately. Guinea pigs without any BCG-treatment but injected with L_2C-leukocytes remained negative; the immunotherapy group became positive and both BCG-vaccinated groups remained positive but generally with a stronger skin reaction than measured in the first test or as compared to that in the immunotherapy group. This enhancement in skin reactivity was expected in repeated tuberculin tests. Some BCG-vaccinated guinea pigs, which received L_2C-leukocytes without BCG, were examined during the whole incubation period until death. These animals were strongly positive all the time. This includes leukemia up to nine weeks after BCG-vaccination. This is in remarkable contrast to the tuberculin conversions observed in leukemic animals during rechallenge tests mentioned above.

Experiment 7: BCG-immunotherapy with enhanced BCG-mass to counteract detrimental effect of preceding BCG-vaccination

In the following approach we tried to restore the antitumor activity of BCG in pre-sensitized guinea pigs by raising BCG concentration mixed with L_2C-leukocytes. The test performed was very similar to that outlined in Fig. 4. The same kind of controls were chosen. Guinea pigs of two groups, ten animals each, were vaccinated by injection 5.0×10^6 CFU of BCG intradermally. One group of 10 animals determined for the positive control of immunotherapy was not vaccinated. Some weeks later 1.3×10^5 L_2C-leukocytes were injected at the beginning of the experiment, two groups in mixture with 2.0×10^8 CFU of BCG. This elevated BCG dose did not improve life expectation of the pre-sensitized group to the level of that of the unvaccinated immunotherapy (data not shown). Comparing both vaccinated groups, addition of the large amount of BCG to unaltered leukemic load brought only a slight advantage ($P=0.025$) in life duration, fewer than noticed in Fig. 5 illustrating an experiment with a normal BCG-concentration of 1.0×10^7 CFU. So an elevated BCG-mass is not able to compensate for the reduction of antitumor activity by pre-sensitization with the same *Mycobacteria*.

In the last three experiments we observed that ulceration of an intradermal leukemic nodule developing during L_2C-incubation period occurred earlier in animals pre-sensitized with BCG than in controls not vaccinated. Intensity of ulceration or number of animals affected did not change with pre-sensitization. Possessing values in three experiments at the same point in time, 12 days after L_2C-inoculation, it was possible to quantify our observation knowing that three weeks after injection of BCG-L_2C-mixture an open ulceration develops in nearly all animals with or without pre-sensitization. The percentages listed in Table 2 clearly show that the development of ulceration is terminated first in pre-sensitized animals. This means that the speed of the local BCG-induced attack against leukemic tumor nodules is accelerated by BCG-vaccination some weeks before. As expected, increase of BCG-mass added to tumor cells injected also accelerate ulceration in guinea pigs not pre-sensitized.

DISCUSSION

BCG-vaccination as a prophylaxis against human leukemia has recently been discussed, but it seems difficult to prove its efficacy statistically [45]. In animals the spontaneously developing leukemia of AKR-mice was somewhat retarded in age-distribution but not reduced in incidence by prophylactic application of BCG [16, 17]. In our study vaccination of guinea pigs with BCG seven weeks before injecting a lethal dose of L_2C-leukocytes did not confer statistically significant protection (Fig. 5). One vaccinated animal, however, survived 102 days after inoculation of tumor cells. Such a long survival time has never before been observed in unvaccinated guinea pigs among a total number of nearly 100

Table 2. Portions of guinea pigs with open ulceration 12 days *after intradermal injection of L_2C-leukocytes plus BCG. Enhancing effect of pre-sensitization with BCG*

Data derived from	CFU of BCG injected i.d. together with L_2C-Leukoc.	Animals with ulceration/ Animals L_2C-inoculated	
		Without pre-sensitization	Pre-sensitized with BCG
Expt. 5	1×10^7	0/11	7/11
Expt. 6		3/10	10/10
Expt. 7	2×10^8	6/10	10/10

Data listed stem from the experiments described in detail above. In all three cases animals got nearly the same load of 1.0×10^5 L_2C- leukocytes in mixture with different quantities of BCG as indicated.

animals. This observation is similar to that described for infection of BCG-vaccinated marmosets with *Herpesvirus saimiri* [46]: again no statistically significant difference from the unvaccinated group was detectable but one animal showed a remarkable delay in development of malignant lymphoma and another one survived infection unaccountably.

A general theory about the mechanism of action of BCG in immunotherapy of malignancies has recently been discussed [47, 48]. In a very concise form the main points are: the initial step is a lymphocyte mediated local macrophage activation by BCG resulting in a non-specific killing of tumor cells. This event was called the "innocent bystander" effect of BCG [49]. In the following phase a release of tumor antigens from destroyed cells occurs enabling the induction of a systemic specific antitumor immunity. Close contact between BCG and tumor cells seems to be essential for the initiation of this sequence of events as demonstrated by using different sarcomas of rat [50, 51], a hepatocarcinoma of guinea pig [36], a murine leukemia [12] and our cavian leukemia as shown in Fig. 5 where BCG injected separately from tumor cells was ineffective.

For some solid tumors the "innocent bystander" effect of BCG is sufficient to eliminate neoplastic disease without the help of systemic immunity. In certain experimental models antitumor immunity was not induced by BCG for unknown reasons or because of a deficiency of the immunologic apparatus. This was demonstrated with six different tumors in mice [49, 52] and with one, P8, out of three tumors in rats [53]. As far as tested, artificial blocking of immunoresponsiveness does not prevent successful abolition of these tumors by BCG [49, 53]. Furthermore, the athymic "nude" mouse suppressed rat sarcoma cells after local employment of BCG [54] proving that systemic immunity is not involved in this process. In one case, the P7 osteosarcoma of rats, BCG has induced antitumor immunity after destruction of neoplastic cells [51], but this immunity was a superfluous, secondary effect because the local effectivity did not require an immunocompetent organism [53]. Exceptions were the CC5-rat osteosarcoma and the cavian hepatocarcinoma where BCG must induce antitumor immunity to be efficient in immunotherapy [37, 51]. However, it seems that antitumor immunity is generally of minor importance in the rejection of most localized solid tumors by BCG.

This is not the case for experimental leukemias although most investigators did not check the immunological state of their "cured" animals after immunotherapy. BCG-induced antitumor immunity was observed for the LSTRA-leukemia of mice [12] and in our experiments for the L_2C-leukemia by demonstrating a resistance against tumor challenge in 83% of animals, which survived by BCG-immunotherapy. Furthermore, these results and several other reports suggest that in leukemia systems BCG acts by establishing systemic antitumor immunity exclusively. During the incubation period, after intradermal application of L_2C-leukocytes until outbreak of leukemia, a nodule develops at the site of injection very similar to a localized tumor. Excision of this

nodule did not prevent the animal from break out of leukemia [19] or alter the course of the disease [20]. These earlier findings were confirmed by the fact that elimimation of the intradermal tumor nodule by BCG-induced ulceration has no effect on following L_2C-leukemia (conclusion of experiment 2). Both experiments, surgical excision and ulcerous destruction, demonstate that the determination for leukemia must occur before the development of a tumor nodule. To evaluate the duration of this decisive stage, which we will call "latency period" in the following, it is important to know that the time required for completion of tumor killing by BCG is seven days or more in a murine sarcoma system [55]. BCG admixed to L_2C-leukocytes and injected intradermally should possess similar restrictions to destroy the malignant cells locally. To build up immunity against L_2C-leukemia by application of irradiated leukocytes requires a minimum of eight days [20]. If systemic immunity is the only weapon against leukemia, BCG very probably acts during latency by killing tumor cells without any possibility of preventing leukemia by this kind of activity. For protection from leukemia a systemic immunity is required, which develops slowly as a consequence of the indolence of BCG to stimulate the immunologic apparatus. Therefore, it is difficult to block induction of leukemia because a sufficient level of systemic immunity has been reached during latency. Only in this case will animals survive with an acquired systemic immunity against leukemia. At present, the mechanism of leukemia induction during latency is unknown. Since tumor viruses were discussed in the L_2C-system [56], it is possible that these agents are introduced or derepressed by the transplanted L_2C-leukocytes causing the disturbance of the hematopoetic system in the recipient. This idea is in agreement with the observation made by others for the E♂G2 murine leukemia [10], namely that an early leukemia related unknown event determines in an almost absolute fashion the efficacy of the BCG-immunotherapy. This fundamental difference between the BCG-immunotherapy of solid tumors and that of leukemias is supported by further results.

Firstly, injecting a mixture of leukemic cells and BCG we got a continuous spectrum ranging from animals which died at the same time as control animals without BCG to guinea pigs with an extremely long delay in onset of the illness or animals resistant against leukemias (Fig. 2). We have never had survivors without observing at the same time a broad gradation of this type. This would be the consequence expected for a competition between BCG and leukemia in individual animals.

Secondly, BCG injected in leukemic nodules just before onset of the disease or in early stages of neoplasia was useless (lowest panel of Fig. 2). Determination for leukemia during latency had expired before BCG was employed. In a solid tumor of the same guinea pig inbred strain, however, injection of BCG into the intracutaneous tumor nodules caused a definite tumor remission in 69% of animals treated [38].

Thirdly, pre-sensitization of guinea pigs with BCG interfered with the immunotherapy of BCG some weeks later (experiments 5 and 6). It is unlikely that in tuberculin positive guinea pigs an immunologic defense mechanism hinders BCG in its "innocent bystander" effect, for example by killing most *Mycobacteria*, because improvement of bacterial mass from 1.0×10^7 to 2.0×10^7 CFU injected did not restore immunotherapeutic effects to a level comparable to that observed in animals not vaccinated with BCG before (experiment 7). Since the local attack of macrophages against adjoining tumor cells seems to be of the same kind in solid tumors as in leukemic nodules, it is informative that pre-sensitization with BCG has no effect upon the successful treatment of solid tumors in mice [52] or guinea pigs [38, 39]. Therefore, in our system BCG-vaccination might interfere in some way with the establishment of systemic antitumor immunity during immunotherapy. In this phase ulceration of the intradermal nodules of leukemic cells appeared earlier in pre-sensitized animals than in guinea pigs not BCG-vaccinated (Table 2). Therefore. one may hypothesize that destruction of immunobilized L_2C-leukocytes and hence, the antigenic stimulus was achieved too early in relation to BCG-stimulation of the reticuloendothelial system.

An additional result of the challenge of guinea pigs surviving by BCG-immunotherapy was the observation that a close relationship exists between the outbreak of neoplastic disease and the alteration of skin reactivity against tuberculin from positive to negative (Table 1). Three of these animals were not resistant against strong L_2C-challenge and died of leukemia with simultaneous loss of delayed hypersensitivity reactivity. A limited systemic immunity against leukemia was nevertheless present in these animals as indicated by prolonged survival times in comparison to those of challenge control animals. Two L_2C-resistant guinea pigs showed a transient leukemia with spontaneous remission

never found in L_2C-susceptible guinea pigs [34]. In both cases leukemia was accompanied again by a temporal but total loss of tuberculin reactivity which was constantly present in all other L_2C-resistant guinea pigs receiving the same tumor inoculum. This conversion of delayed hypersensitivity would not have been caused by an immunosuppressing exogenous infection, repeated skin testing or ageing of reactivity against tuberculin as supported by appropriate controls and maintained by known literature [27, 57]. This disappearance of delayed hypersensitivity reaction is a further distinction from the hepatocarcinoma system in guinea pigs where progressive growth of BCG-adjoining tumor cells was not preceded by a loss of skin reactivity to tuberculin [40]. Therefore, in the guinea pig leukemia system loss of skin reactivity reflects at least partially break down of immunologic surveillance against L_2C-leukemia. The mechanism of this strong connection between tuberculin reaction and immunologic surveillance against leukemia must remain unclarified.

Another question left unanswered is the self-restriction of leukemia by a BCG-initiated immunological defense mechanism. This is the central problem in immunotherapy of leukemia, namely to strengthen the biologic barrier of the host against tumor recurrence. The availability of the L_2C-leukemia for BCG-experiments may now help investigators understand the process preceding the relapse of patients free of detectable leukemia after chemotherapy.

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Enzymes, Lymph Node Involvement and Possible Prognosis in Human Breast Cancer

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Abstract—*The activities of seven enzymes associated with carbohydrate metabolism were measured in tumours from patients with primary carcinomas and the results were analysed in three different ways, i.e. according to tissue weight and enzyme/6PGDH and enzyme/PFK ratios. The tumours were then grouped together according to the number of nodes found to contain metastases and the following significant differences were observed. 1. Significantly lower activity of α-GPDH was observed in tumours from patients in whom four or more lymph nodes were found to contain metastatic deposits than in those with no nodal involvement. 2. Enzyme/6PGDH and enzyme/PFK ratios were significantly lower in tumours from patients with four or more nodes involved than in those with no nodal involvement. Possible prognostic significance of these enzymes is discussed.*

INTRODUCTION

IT IS generally accepted than an early indication of the clinical course of any carcinoma will greatly assist clinicians in deciding on a suitable treatment. A considerable effort therefore is being made to devise biochemical parameters for this purpose. It is hoped that carcinomas will either secrete specific factor(s) which are not present in non-cancerous women or alter the body pool concentrations of parameter(s) normally present so that there are significant differences between the two categories. These hypotheses are then extended in clinical studies to test whether such parameter(s) can correlate with the prognosis. In no field is this more relevant than in carcinoma of the breast. Although the ideal factor(s) for this purpose has not been observed, several workers have attempted to investigate hormones, tumour antigens, proteins and enzymes as likely candidates and found some abnormalities in patients with breast carcinoma [1–4]. In recent studies activities of enzymes associated with carbohydrate metabolism in primary breast carcinomas have been measured to test the hypothesis that the energy requirements of a rapidly growing carcinoma may be related to the rate of glycolysis and therefore by measuring the activities of certain enzymes it might be possible to predict the clinical course of the disease [5–7]. If so, they should correlate with the recurrence and survival rates. It is well known that in women with primary breast cancer, the disease-free interval and 5 yr survival is greatly reduced if lymph nodes are found to contain metastatic deposits at the time of mastectomy [8, 9]. Therefore, analysis of the results obtained on enzyme activities in the primary carcinomas related to the presence or absence of nodal involvement at the time of mastectomy might give some insight into the usefulness of these measurements in prognosis. This paper reports the findings after such an analysis.

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MATERIAL AND METHODS

All the chemicals used in the study were purchased from British Drug Houses Ltd. and were of analytical grade. α-GPDH, TPI, G6PDH, aldolase, NADH, $NADP^+$, ATP, F-6-P, G-6-P, 6PG and pyruvate were purchased from Boehringer Mannheim. G-1-P was purchased from Koch-Light Ltd. DHAP was prepared from the dicyclohexylammonium salt of the dimethylketal according to the manufacturer's (Boehringer Mannheim)

instructions.* Primary tumours were obtained from patients with carcinoma of the breast undergoing mastectomy. The malignancy of the tumours was confirmed by histological examination. The tissues were wrapped in aluminium foil, frozen on solid carbon dioxide and transferred to the laboratory where they remained frozen (−20°C) until further processing. All the tissues taken for histology were processed routinely and section from two levels of each lymph node block examined in order to increase the chances of finding small deposits of metastatic tumour. In this series an average of 23 axillary lymph nodes (range 8–58) were found in radical mastectomy specimens.

The tissues were cleared of any surrounding fat, weighed and cut into small pieces. The semi-frozen tissues were then homogenised in a Silverson homogeniser according to the procedure of Shonk and Boxer [10]. The homogenates were centrifuged (800 ***g*** for 15 min) at 4°C, the cytosol fraction was decanted off and used as a source of enzymes. A 5% solution of tumours was used in these estimations. The activities of PFK, G6PDH, 6PGDH, PGM, LDH, α-GPDH and PHI were measured. Enzyme assays were performed in a Beckman recording spectrophotometer according to the procedure of Shonk and Boxer [10], with the exception that one minute readings were recorded automatically for five minutes and a mean of the last four was taken as an average change in optical density (O.D.).

The results were calculated on the basis of units (U) of enzyme activity per gram wet weight of tissue. A unit is defined as that amount of enzyme which will catalyse the transformation of 1 μmole of substrate per minute. The results were analysed in three different ways: as units of enzyme activity/g and as enzyme/6PGDH and enzyme/PFK ratios. They are presented as mean ± S.D. The means were tested for significance by a Student's '*t*'-test.

*The following abbreviations are used in this paper.
Enzymes—α-GPDH, α-glycerolphosphate dehydrogenase; TPI, triosephosphate isomerase; G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructo kinase; 6PGDH, 6-phosphogluconate dehydrogenase; PGM, phosphogluco mutase; LDH, lactate dehydrogenase; PHI, phosphohexose isomerase.
Co-enzymes—NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.
Substrates—F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; 6PG, 6-phosphogluconate; G-1-P, glucose-1-phosphate; DHAP, dihydroxyacetone phosphate.

RESULTS

This study is based on a total of 95 patients, 47 of these were free of nodal involvement. Of the remainder there were 10 patients with only one involved lymph node, 8 with two, 3 with three and 27 in whom four or more nodes were involved. For the purpose of analysis, patients with two or three involved nodes were grouped together.

The values (U/g) obtained in these four categories are shown in Table 1.

There were no statistically significant differences in the activities of G6PDH, 6PGDH, PGM, LDH and PHI between patients with no node involvement and those in the one, 2+3 or 4+ categories. α-GPDH activities were significantly lower in patients in the 4+ category than in those in whom no nodes were found to contain metastases.

Further analyses of the results on the basis of enzyme/6PGDH and enzyme /PFK ratios are shown in Tables 2 and 3, respectively.

In both cases significantly higher α-GPDH/6PGDH and α--GPDH/PFK ratios were observed in tumours from patients with no nodal involvement than those in whom four or more nodes were found to contain metastatic deposits. Furthermore, α-GPDH/PFK ratios in tumours from patients who had two or three involved nodes were higher than those with four or more involved nodes.

DISCUSSION

Recurrence and survival after mastectomy are usually considered to be dependent on certain clinical factors such as size of carcinoma, histology, anatomic type, grade of malignancy and lymph node involvement. The most closely related amongst these variables was the spread of the disease to the surrounding lymph nodes. Fisher and his colleagues [8] have shown that after surgical removal of the breast the 5 yr survival rate in patients with localised disease and absence of nodal involvement is 76% which drops to 45% when up to three nodes are involved and 31% when four or more are found to contain metastatic deposits. The same also applies to recurrence rates. In the absence of axillary metastases the 5 yr recurrence rate is 21%, which rises to 81% if four or more nodes are involved. Similar findings have been reported by others [11, 12]. However, these are gross classifications and as has been pointed out by Cutler and Myers [11] the patients falling into the same prognostic class show marked variation in clinical characteristics. Therefore, further classification

Table 1. *Enzyme activities* (μmole *of pyridine nucleotide reduced or oxidised*/min/g *tissue*) *in human breast carcinomas. The tumours were separated according to the number of lymph nodes found to contain metastatic deposits*

Enzyme ↓ / Nodes →	0	1	2+3	4+
PFK	0.057+0.044*	0.093±0.057	0.060±0.040	0.094±0.079
G6PDH	0.197±0.149	0.212±0.110	0.150±0.090	0.178±0.119
6PGDH	0.132±0.091	0.134±0.064	0.130±0.070	0.137±0.065
PGM	1.396±0.829	1.466±0.698	1.560±0.780	1.339±0.688
LDH	7.019±4.781	6.939±3.072	8.950±6.530	8.582±3.982
α-GPDH	0.310±0.249*	0.214±0.192	0.260±0.130	0.182±0.168
PHI	7.859±4.337	8.027±3.655	7.270±3.680	8.868±4.406

0—No nodes involved; 1—One node involved; 2+3—Two or three nodes involved; 4+—Four or more nodes involved.

*Significant differences between 0 and 4+ $P<0.02$.

Table 2. *Enzyme/6PGDH ratios in human breast carcinomas. The tumours were separated according to the number of lymph nodes involved*

Enzyme ↓ / Nodes →	0	1	2+3	4+
PFK	0.629± 0.453	0.701± 0.258	0.550± 0.449	0.751± 0.487
G6PDH	1.555± 0.754	1.660± 0.800	1.375± 0.587	1.342± 0.817
6PGDH	—	—	—	—
PGM	11.330± 4.454	11.267± 4.041	13.020± 6.538	12.123± 6.227
LDH	58.287±26.726	51.547±21.038	53.132±14.755	67.079±25.972
α-GPDH	2.495±2.400*	1.622± 1.026	3.024± 2.476	1.248± 1.067
PHI	71.488±24.125	63.542±22.009	67.846±20.653	75.839±25.301

*Significant differences between 0 and 4+ ($P<0.01$).

Table 3. *Enzyme/PFK ratios in human breast carcinomas. The tumours were separated according to the number of lymph nodes involved*

Enzyme ↓ / Nodes →	0	1	2+3	4+
PFK	—	—	—	—
G6PDH	3.083± 2.305	2.227± 0.994	2.415± 1.165	2.878± 2.629
6PGDH	2.506± 1.685	1.588± 0.512	2.672± 1.392	1.876± 1.386
PGM	26.312± 15.404	17.540± 8.018	26.993± 11.123	21.857± 12.221
LDH	147.311±104.179	95.215±52.796	157.431± 79.930	148.184±121.812
α-GPDH	4.254± 4.302*	1.993± 1.384	4.957± 4.458†	1.441± 0.819
PHI	142.964± 80.724	113.730±37.165	157.176±103.139	113.372± 58.223

*Significant differences between 0 and 4+ ($P<0.01$).
†Significant differences between 2+3 and 4+ ($P<0.001$).

on other indices is desirable. It is possible that biochemical parameters, in conjunction with clinical variables, may find a role in further separation of likely candidates who might require early adjuvant therapy.

Although a large number of parameters have been investigated in the hope that they could be used as prognostic aids, none is known to be in routine clinical use and with two exceptions they have not been found to correlate with recurrence rates [13, 14]. In this study we have attempted to ascertain whether enzyme patterns in these tumours correlate with other factors related to prognosis. To correlate with prognosis, the para-

meter must show significant differences between known clinical variables. In this respect the results are showing some promise in that lymph node involvement which is associated with poor prognosis, shows differences in both enzyme activities and ratios. In particular α-GPDH activities and α-GPDH/6PGDH and α-GPDH/PFK ratios show significant differences between tumours from patients in whom no nodes were found to contain metastatic deposits and those in whom four or more nodes were involved. It remains to be seen whether these patients with low α-GPDH activity will develop recurrences sooner irrespective of nodal involvement. If so, the α-GPDH activity or α-GPDH/6PGDH ratio might prove to be a useful parameter in predicting the clinical course of the disease.

Low levels of α-GPDH have been noted in all the neoplastic tissues [15]. However, the results reported here indicate that the activity of the enzyme is significantly different between tumours from patients with no nodal involvement and those in whom four or more nodes were involved. Although it is known that in some animal tumours growth rates correlate with the activity of glycolytic enzymes, it is not clearly understood what regulates the relative activities of enzymes within tissues [16]. In hormone responsive tissues such as carcinogen-induced rat mammary tumours or epididymis, oophorectomy and testosterone treatment appear to alter enzyme levels respectively [17, 18]. But these are hormone dependent tissues and withdrawal of or treatment with the hormone is bound to induce major changes in growth which are reflected in enzymic activities. Whether the enzyme values reported here correlate with the endocrine status of these patients remains to be explored.

In conclusion, certain enzyme activities in primary carcinomas appear to correlate with known clinical parameters involved in prognosis. In particular α-GPDH/6PGDH and α-GPDH/PFK ratios show significant differences between tumours from patients with no nodal involvement and those in whom four or more nodes were found to contain metastatic deposits.

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Isoenzymes of Hexokinase in the Developing, Normal and Neoplastic Human Brain*

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Abstract—*The activities and isoenzyme distributions of soluble hexokinase were investigated spectrophotometrically and by cellulose acetate membrane electrophoresis in extracts of normal human brain, a series of fetal brains ranging in gestational age between* 6 *and* 42 *weeks and in* 27 *gliomas. The enzyme was first detected at* 14 *weeks gestational age, at a level approximately half of the normal adult level. Only one isoenzyme (type* I*) was present. The activity gradually increased until similar levels to those found in the adult were reached at term. The rise in enzyme levels was particularly rapid between* 35 *and* 40 *weeks. This rapid rise was associated with the appearance of a second isoenzyme of hexokinase (type* II*), first detected at the age of* 36 *weeks gestation. Normal adult brain contained type* I *hexokinase only. Further work needs to be done to determine when the type* II *enzyme disappears.*

Types I *and* II *isoenzymes were detected in fourteen of the gliomas studied, with a high incidence in the poorly differentiated tumours.*

INTRODUCTION

HEXOKINASE (ATP: D-Hexose-6-phosphotransferase. EC 2.7.1.1.) has been shown to exist in multiple molecular forms in mammalian tissues. Four isoenzymes have been identified by chromatographic [1] and electrophoretic [2] techniques. They have been numbered from I–IV in order of increasing mobility towards the anode. The distribution of types I–III has been shown to vary from tissue to tissue, at different ages and with the nutritional status of the animal [3–7]. Type IV hexokinase is the specific glucokinase [8, 9] and has been found only in liver and kidney [3–7, 10]. Mammalian brain has been shown to contain mainly type I hexokinase with a trace of the type II enzyme [5–7].

Studies of hexokinase in neoplasia have been mainly confined to experimental rat hepatomas. Several groups have demonstrated a progressive loss of glucokinase and an increase in hexokinase with increasing tumour malignancy [11–16]. Recently variations in the isoenzyme pattern have been observed in human uterine tumours [17, 18].

The present paper is a study of the activities and isoenzyme patterns of soluble hexokinase in normal adult human brain, in a series of 31 fetal brains ranging in gestational age from 6 to 42 weeks and in 27 gliomas.

MATERIAL AND METHODS

Glucose-6-phosphate dehydrogenase (G-6-PDH) and disodium nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Boehringer Mannheim, West Germany; dithiothreitol (DTT), and phenazine methosulphate (PMS) from the Sigma Chemical Company, London; and all other reagents from British Drug Houses, United Kingdom. All reagents were of the purest grade available. "Celagram" cellulose acetate membranes (78 × 150 mm) were obtained from Shandon Scientific Company, United Kingdom.

Normal human cerebral tissues were either obtained at autopsy within 15 hr of death, or directly after surgical removal. The cases analysed ranged in age from 17 to 50 yr.

Fetal brains up to 28 weeks gestation were obtained from spontaneous or induced abortions and fetal brains between 28 and 42 weeks

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gestation were obtained from post mortem specimens. None of these post mortems showed any evidence of cerebral abnormalities.

Samples of tumours of the nervous system were obtained directly after surgical removal.

All tissues were either used immediately or stored at −20°C until used. No changes were observed in enzyme activity or in isoenzyme patterns after storage of whole tissues or soluble cell fractions at −20°C for up to one month.

Samples were homogenised over ice in a bench homogeniser (Silverson U.K.) for two periods of 30 sec with a 1 min interval. The tissues were homogenised in 100 mM potassium phosphate buffer pH 7.4 containing 2 mM EDTA and 0.2 mM DTT. The crude homogenates were centrifuged at 20,000 × *g* for 45 min and the clear supernatant containing the soluble cell fraction filtered through gauze. Part of this fraction was made up to a final dilution of 1 in 10 with homogenizing buffer for enzyme assay. The remainder was made up to a final dilution of 1 in 4 prior to electrophoresis.

Hexokinase was assayed by coupling the enzyme reaction to the reduction of NADP by the G-6-PDH reaction and following the change in absorption at 340 nm in an SP1800A recording spectrophotometer (Pye Unicam, U.K.) with a constant cell temperature of 37°C. The assay system was essentially that of Beutler [19] and consisted of a 10 min preincubation of the enzyme preparations in 0.1 M Tris/HCl buffer pH 8.0 containing 2.0 mM D-glucose, 0.2 mM NADP, 0.2 units/ml of G-6-PDH and 3.0 mM magnesium chloride. The reaction was initiated by the addition of ATP at a final concentration of 3.0 mM. Addition of 0.2 units per ml of 6-phospho-gluconate dehydrogenase caused no further increase in enzyme activity.

Total protein concentrations were measured by the automated modification of the biuret reaction [20].

A unit of enzyme activity is defined as the amount of enzyme which will convert 1 μmole of D-glucose to glucose-6-phosphate/min/mg protein under the assay conditions at 37°C.

Electrophoretic procedure was adapted from a previously reported method [18]. Eight samples of approximately 0.1–0.2 munits of enzyme activity were run side by side on each cellulose acetate membrane using a buffer concentration of 50 mM barbitone, 5.0 mM EDTA, 1.0 mM DTT and 10.0 mM D-glucose at pH 8.6. A total potential of 28.2 V/cm was applied for 1 hr by which time a marker of albumen-bound bromophenol blue had migrated 4.8 cm towards the anode. The isoenzymes were localised *in situ* by placing the membrane in contact with a 0.5% agarose gel containing 50 mM Tris, 5.0 mM ATP, 5.0 mM $MgCl_2$, 2.0 mM KCN, 0.5 mM NADP, 25 μg/ml PMS, 0.4 mg/ml nitro blue tetrazolium, 0.2 units/ml G-6-PDH and 10.0 mM D-glucose at a final pH of 8.2. Incubation was carried out at 37°C for 1 hr in the dark after which time the insoluble blue bands associated with enzyme activity could be seen. The membranes were fixed in 5% acetic acid for 15 min and scanned at 578 nm with a vitatron densitometer (Fisons, U.K.).

Controls were set up using identical electrophoretic conditions but in this case the membranes were stained in a medium lacking substrate (D-glucose) and no bands of enzyme activity were seen on any of these membranes.

RESULTS

The pattern of development of hexokinase activity in fetal brain is shown in Fig. 1. The enzyme was first detected at the gestational age of 14 weeks and at this time the level was approximately half that found in the normal human adult brain. This level showed a gradual increase throughout gestation reaching the adult level at term.

There was no significant difference in the activity of hexokinase in adult grey and white matter in material obtained at autopsy, but the activity in grey and white matter was slightly higher in fresh surgical specimens than in grey and white matter obtained at autopsy. Mean hexokinase activity in autopsy specimens was 0.0030 μmole/min/mg protein ($N=10$, S.D. $=0.0018$) whilst that for surgically removed specimens was 0.0038 μmole/min/mg protein ($N=28$, S.D. $=0.0016$). This indicates a slight loss of activity during autolysis.

Electrophoresis of fetal and adult brain homogenates showed that during the first 36

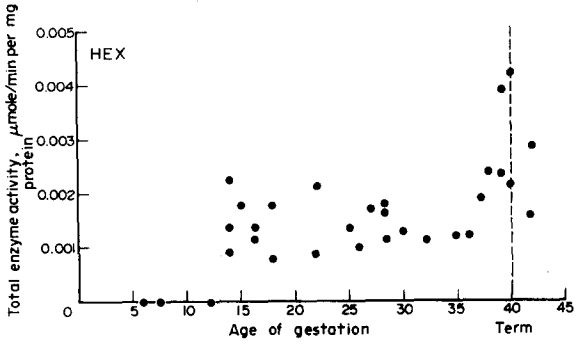

Fig. 1. The development of hexokinase activity in fetal human brain. Extracts of fetal brain were prepared and assayed spectrophotometrically by the method described in the text. The corresponding adult mean values are 0.0030 μmole/min/mg *protein* (units/min/mg *protein*) *for autopsy specimens and* 0.0038 μmole/min/mg *protein for surgical specimens. The horizontal axis is gestational age in weeks.*

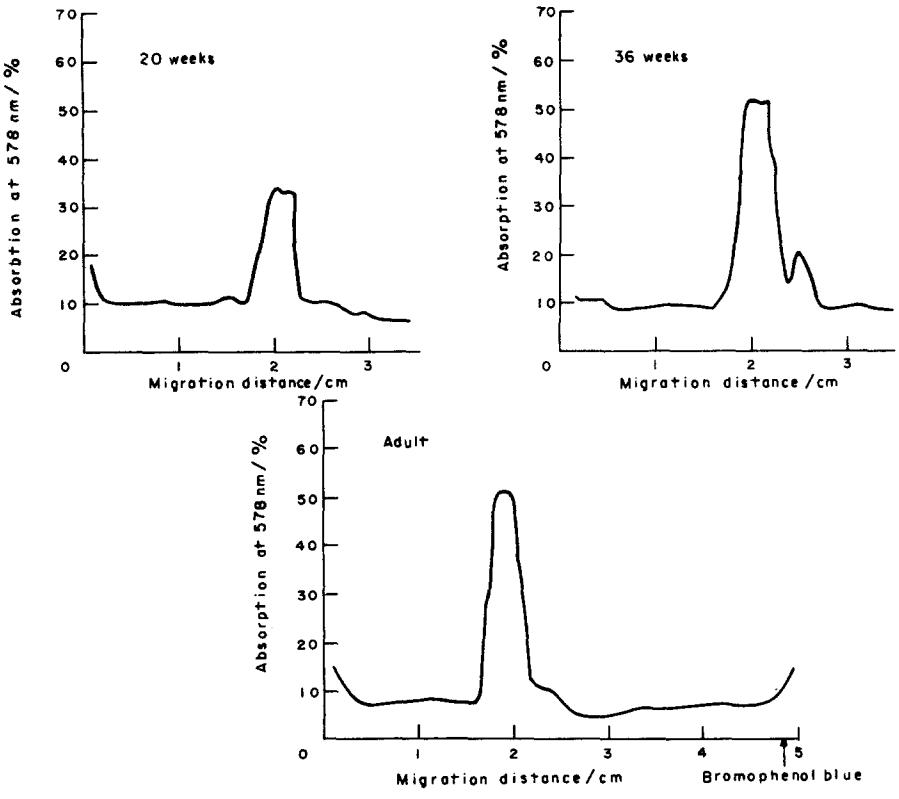

Fig. 2. Scan profiles at 578 nm *of fetal and normal adult cerebral tissues subjected to cellulose acetate membrane electrophoresis for one hour by the method described in the text, and stained for hexokinase activity* in situ.

Table 1.

Tumour type	No. of tumours with type II isoenzyme detected	Percentage of activity defined by relative peak area		Enzyme activity
		Type I	Type II	μmole/min/mg
Astrocytoma grade IV $N=15$	10	87±14%	13±14%	0.0035±0.0014*
Astrocytoma grade III $N=6$	1	98± 4%	2± 4%	0.0029±0.0018*
Astrocytoma grade II $N=4$	2	98± 3%	2± 3%	0.0022±0.003 *
Ganglioglioma $N=1$	0	100	0	0.0072
Ependymoma	0	100	0	0.0023

*Standard deviation.

weeks of gestation and in adult specimens, only one band of hexokinase, type I, was present, whilst during the last 4–6 weeks of gestation two isoenzymes, types I and II, were consistently observed (Fig. 2).

In an electrophoretic survey of 25 astrocytomas (15 grade IV, 6 grade III and 4 grade II), type II hexokinase was seen in 10 of the grade IV astrocytomas, in one of the grade III astrocytomas and in 2 of the grade II astrocytomas. The more benign gliomas, including two of the grade II tumours, a single ganglioglioma, and a well-differentiated ependymoma, showed an identical pattern to that of normal adult brain. Electrophoresis was carried out on samples showing approximately similar activities of enzyme and the relative proportions of each isoenzyme present were calculated by integration of the respective peak areas after scanning (Table 1).

DISCUSSION

Between 15 and 44% of hexokinase in the mammalian brain has been shown to be mitochondrial-bound [21, 22]. It has also been suggested that the soluble hexokinase in brain is largely of glial cell origin and that the particulate fraction is neuronal [23]. In the present study only soluble hexokinase was measured and the results obtained could bear a direct relationship to the glial cell content. All tumours studied were glial cell in origin.

The study has shown that the soluble fraction of hexokinase, the major glycolytic rate limiting enzyme in brain [24] appeared in significant quantities at about 14 weeks gestational age. Other glycolytic enzymes including aldolase, pyruvate kinase, phosphofructokinase and phosphoglucomutase have also been shown to appear at this age in man [25]. Since soluble hexokinase may be a marker of glial cells in brain this period may coincide with an important period of glial cell development.

Electrophoretic studies show that only type I hexokinase was present in fetal brain up to 36 weeks gestational age when the type II isoenzyme appeared. This enzyme is not present in the normal adult human brain and the significance of its appearance late in fetal development is unclear. There is clearly a need for further work on this enzyme.

Type II hexokinase was also found in many of the gliomas studied. The highest incidence was seen in the more malignant tumours (grade IV). The presence of this enzyme in neoplastic and fetal brain and its absence in adult brain implies that the isoenzyme is a carcino-embryonic antigen, one of a large number that have now been described [26, 27]. The appearance of the enzyme does not signify a dedifferentiation towards primitive undifferentiated forms since the isoenzyme was only observed in the later stages of development when the tissues are showing evidence of differentiation. It seems that this reversion is towards a highly specialised period of cerebral development implying an ordered alteration in genetic activity in this particular case. The fact that the isoenzyme form appeared predominantly in the more malignant tumours implies that this particular misprogramming of genetical information is a consequence of neoplasia and not a cause of it.

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Phase II Study of Anhydro-Ara-5-Fluorocytidine in Adenocarcinoma of Gastrointestinal Tract, Epidermoid Carcinoma of Lung, Head and Neck, Breast Carcinoma and Small Cell Anaplastic Carcinoma of Lung*

A Study Report of the E.O.R.T.C. Early Clinical Trial Cooperative Group

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Abstract—*AAFC is a fluorinated anhydride analog of cytosine arabinoside, partially hydrolysed* in vivo *into ara-FC and ara-FU. Antitumor activity has been observed during phase* I *studies in acute leukemia and solid tumors.*

A hundred and sixty six patients with advanced tumors belonging to selected histological types received AAFC, 15–20 mg/kg *daily* × 5 *q3w, or* 33–40 mg/kg *weekly i.v. during* 3 *weeks or longer. One hundred and thirty-two observations were fully evaluable including* 67 *without and* 65 *with prior chemotherapy. Responses were observed in adenocarcinoma of stomach* (7/14), *pancreas* (2/3) *and colon and rectum* (2/32), *epidermoid carcinoma of head and neck* (4/30), *breast carcinoma* (1/17) *and small cell carcinoma of lung* (1/9), *but not in epidermoid carcinoma of lung* (0/27). *Both treatment schedules were active. There were* 8/67 *responders among not pretreated and* 9/65 *among pretreated patients.*

Toxicity was more pronounced after daily × 5 *application. The limiting toxicity consisted in anemia, leucopenia, thrombopenia, nausea and vomiting. Parotid pain or postural hypotension were not observed.*

INTRODUCTION

ANHYDRO-ARA-5-FLUOROCYTIDINE (2,2′-anhydro-arabino-5-fluorocytosine, AAFC, NSC-166641) is a new arabinosylcytosine (ara-C) derivative with pharmacological features suggesting a possible superiority to ara-C as an antineoplastic agent. Like other similar cyclo- or halogenated compounds it is more resistant to deamination than ara-C resulting in a longer half life in blood [1]. This point is of clinical importance, considering the S-phase specific properties of ara-C and ara-C derivatives. The physiological disposition of AAFC is characterized by a conversion to arabinosyl-fluorocytosine (ara-FC) and arabinosyl-fluorouracil (ara-FU), two active antitumor substances [2]. Moreover, it has been postulated that AAFC could be active on its own, being mainly excreted unconverted and more active than ara-C in ara-C resistant leukemia-cell lines [3]. Finally, it has been demonstrated that, contrary to ara-C, AAFC can be given at a therapeutically

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‡All participating investigators and institutions are mentioned in Table 1.

active dose by single weekly i.v. injections and that its use is not limited, as for cyclocytidine [4], by parotid pains or postural hypotension.

AAFC has been shown to be active in human acute myeloblastic leukemia, either alone or in combination [5]. The present report constitutes the first large clinical investigation of the use of AAFC in adult patients with solid tumors. A progress report on 105 cases has already been presented in abstracted form elsewhere [6].

MATERIAL AND METHODS

Selection of patients

The eligibility was restricted to a limited number of tumors. Epidermoid carcinoma of head and neck and of lung and adenocarcinoma of stomach, colon, rectum and pancreas were considered tumor types of first priority because of their poor responsiveness to available agents. Carcinoma of the breast and anaplastic small cell carcinoma of the lung were also considered eligible because of their frequency and of the great number of patients surviving with tumors primarily or secondarily resistant to other available treatments. All other tumor types were rejected, not because they were judged less important, but as a consequence of practical considerations concerning the number of observations statistically required for each tumor type studied. The criteria for inclusion were a histologically confirmed tumor diagnosis corresponding to one of the selected tumor types, measurable and evolutive disease not suitable for surgery or radiation therapy, a Karnofsky performance >40, age >15, granulocytes $>2500/mm^3$, thrombocytes $>100{,}000/mm^3$, blood creatinine $<1.5/100$ ml. Patients with a second tumor, or with overt psychosis, senility or central nervous system lesions were excluded. Pleural effusions, bone and brain metastases were not considered measurable lesions. Patients were not eligible during or immediately after other chemo- or radiation therapy as well as during the first three weeks after the last day of the previous treatment. The only exception was a palliative radiation on a limited bone lesion outside the location of measurable tumor masses.

Treatment schedules

The treatment schedules compared correspond to the ones used in two Phase I studies in solid tumors [7, 8]:

1. AAFC 15 mg/kg body weight daily for five consecutive days by rapid i.v. injections in one daily dose to be repeated every three weeks;
2. AAFC 33 mg/kg body weight in one weekly rapid i.v. injection to be repeated weekly.

The dose was modified according to toxicity or a weekly dose omitted. Anti-emetics were largely used to counteract nausea and vomiting. Whenever possible, the treatment was pursued for 6 weeks or more, unless it was considered in the interest of the patient to stop it earlier. The dose originally scheduled in regimen 1 was 20 mg/kg/day. A severe haematologic toxicity encountered in the first patients in regimen 1 required a downward modification to 15 mg/kg/day. Later during the study, it appeared that even after this dose modification regimen 2 was less toxic and apparently as active as regimen 1 so that this latter was dropped and the dose for regimen 2 raised to 40 mg/kg/week.

Mechanics of the study

Principal investigators and institutions participating in this study as members of the Group are mentioned in Table 1. The study was activated in February 1975 and closed in May 1976. The final analysis was made on follow-up data available as of December 1976. Patients registration and randomization were centralized at EORTC Data Center in Brussels. Patients were randomly allocated to one of the two treatment schedules defined in the above section on a 1:1 rate. Randomization was stratified with regard to institution, tumor type and previous treatment with chemotherapic agents. Treatment results quoted as objective response by local investigators were presented with all available documents at Group meetings and the final assessment of the therapeutic result for each of these cases was made by general agreement of all Group members. All protocol documents were finally reviewed for eligibility, quality of data recording, protocol violations and treatment results by the chairman and the secretary of the Group.

Tumor measurements, drug toxicity, tumor symptoms, weight, temperature, analgesics and other treatments and a complete blood count were registered before treatment and at weekly intervals. Blood creatinine, alkaline phosphatase and transaminases were registered every three weeks.

Evaluation of response

Patients records were considered inevaluable whenever one or more of following reasons were present: ineligibility, incomplete data,

Table 1. *Participating members of the Early Clinical Trials Group (EORTC)*

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Table 2. Number of patients by tumor type and treatment schedule

Tumor type	Evaluable		Not evaluable					Total entered
	Reg. 1	Reg. 2	T.R.	L.F.	I.	P.V.	I.D.	
Epidermoid carcinoma of head and neck	11	19	0	0	0	1	4	35
Epidermoid carcinoma of lung	12	15	1	0	4	3	6	41
Adenocarcinoma of colon and rectum	8	24	0	0	0	1	2	35
Adenocarcinoma of stomach and pancreas	5	12	0	1	2	1	2	23
Breast carcinoma	6	11	0	0	1	0	0	18
Anaplastic small cell carcinoma of lung	3	6	0	1	1	2	1	14
Total	45	87	1	2	8	8	15	166

T.R.: Treatment refused.
L.F.: Lost to follow-up within first 2 weeks of treatment.
I. : Ineligible.
P.V.: Protocol violation.
I.D.: Incomplete data.

improper randomization, major protocol violation, early loss to follow-up (first two weeks of treatment) and treatment refusal. Evaluable observations were classified in five response categories. Complete response corresponds to a complete disappearance of all measurable

tumor lesions. Partial response corresponds to a decrease of more than 50% of the product of the two largest perpendicular diameters of all measurable lesions without appearance of other tumors. In case of metastatic liver enlargement or other deep abdominal tumor masses, a reduction by 75% of the evaluated tumor mass was required. Stable disease corresponds to minor variations within upper and lower 50% limits. Progression corresponds to a 50% or more increase of the product of perpendicular diameters of measurable lesions, or a 75% increase of evaluable tumor masses. Early death corresponds to patients dying within the first three weeks of treatment. In this category, an attempt was made to distinguish between tumor related and treatment related death.

RESULTS

Antitumor effect

One hundred and sixty-six patients were registered and randomized. Thirty-four patients were rejected as being not evaluable: 8 were ineligible, 15 for incomplete data, 8 for major protocol violation, 2 were lost to follow-up and 1 for treatment refusal. Among 132 evaluable patients, 6 were treated at 20 mg/kg × 5 days every 3 weeks, 39 at 15 mg/kg × 5 days every 3 weeks, 53 at 33 mg/kg weekly and 34 at 40 mg/kg weekly. The distribution of patients by cell type is seen in Tables 2 and 3.

AAFC was found to be ineffective in epidermoid carcinoma of lung. A low grade activity was observed in epidermoid cancer of head and neck, with 4 responses in 30 patients, corresponding to a response rate of 13.3% (95% confidence limits of 3.8% and 30.7%) and in adenocarcinoma of colon and rectum with 2 responses in 32 patients or 6.3% (95% confidence limits of 0.8% and 20.8%). AAFC was found active in the group of adenocarcinoma of stomach and pancreas with 9 responses in 17 patients, or a response rate of 52.9% (95% confidence limits of 27.8% and 77.0%). For anaplastic small cell carcinoma of lung, 1 response was observed in 9 patients and for breast carcinoma 1 response in 17 patients (Table 3).

Comparing the treatment schedules, the overall response rate was 3/45, or 6.7% for regimen 1 and 14/87, or 16.1% for regimen 2. Focusing on adenocarcinoma of the stomach and pancreas, the corresponding response rates are 1/5, or 20% for regimen 1 and 8/12, or 66.7% for regimen 2. These differences are not significant at the level of probability $P = 0.05$ (chi square test corrected). There is no difference

Table 3. AAFC: Number of responders/number of evaluable patients

Tumor type	Regimen 1		Regimen 2		Total	%	95% Confidence limits
	No prior treatment	Prior treatment	No prior treatment	Prior treatment			
Epidermoid carcinoma of head and neck	1/7	0/4	0/9	3/10	4/30	13.3	(3.8%, 30.7%)
Epidermoid carcinoma of lung	0/8	0/4	0/12	0/3	0/27	0.0	(0.0%, 12.8%)
Adenocarcinoma of colon and rectum	0/4	0/4	0/10	2/14	2/32	6.3	(0.8%, 20.8%)
Adenocarcinoma of stomach and pancreas	1/5	0/0	6/8	2/4	9/17*	52.9	(27.8%, 77.0%)
Breast carcinoma	0/0	0/6	0/2	1/9	1/17	5.9	(0.1%, 28.7%)
Anaplastic small cell carcinoma of lung	0/1	1/2	0/1	0/5	1/9	11.1	(0.3%, 48.3%)
Total	2/25	1/20	6/42	8/45	17/132	12.9	(7.7%, 19.8%)

*Stomach 7/14; pancreas 2/3.

Table 4. Duration of treatment, remission and survival in responders

Tumor type	Prior chemotherapy	Regimen	Onset of response†	Treatment	Duration* of remission	Survival
Epid. head and neck	No	1	4	6	3‡	7
Epid. head and neck	Yes	2	12	12	16	30‡
Epid. head and neck	Yes	2	6	17	10	71‡
Epid. head and neck	Yes	2	6	6	5	11
Colon and rectum	Yes	2	12	20	11	44
Colon and rectum	Yes	2	5	18	4	31‡
Stomach	No	2	9	18	15‡	23
Stomach	No	2	4	42	55	69‡
Stomach	No	2	4	4	2‡	5
Stomach	No	2	2	4	3	5
Stomach	No	2	5	27	21	31‡
Stomach	Yes	2	3	10	7	26
Stomach	Yes	2	2	5	2	6
Pancreas	No	1	5	8	5‡	9‡
Pancreas	No	2	3	5	3	10‡
Breast	Yes	2	2	3	6‡	7
Small cell lung	Yes	1	4	5	1‡	4

*In weeks from onset of treatment.
†In weeks.
‡Censored observation.

in response rate for patients previously treated (9/65, or 13.8%) with other antitumor agents and for untreated patients, (8/67 or 11.9%). For G.I. tract and pancreas tumor patients responses were observed in 4/22 or 18.2% and 7/27 or 25.9% respectively. Patients with G.I. tumors previously treated did receive FU and were probably resistant to it at the time they entered the AAFC trial.

Duration of response

The median length of remission for responders, defined as the time elapsed between the first evidence of tumor regression and tumor relapse, was 9 weeks (2–55 weeks). Responses were obtained after 2–12 weeks. The median duration of treatment for responders was 8 weeks (3–42 weeks). Because of the great heterogeneity of the patients treated the survival from treatment day 1 varies considerably from 4 weeks to more than 71 weeks, among treatment responders (Table 4).

Toxicity, tolerable dose

The dose limiting toxicity of AAFC consisted of myelosuppression and gastric intolerance. Besides leucopenia and thrombocytopenia anemia was frequent and severe with a loss of hemoglobin concentration reaching 25–35% of the initial figure. Red cell transfusions were required in many cases during and after AAFC treatment. The hematologic toxicity was proportional to the dose (Table 5). Treatment regimens in 5 days were more toxic than weekly injections. Myelosuppression was observed at the time of 5 deaths, caused by sepsis, hemorrage or acute cardiac failure. Nausea and vomiting appeared frequently during the first few hours after injection. Their intensity appeared not to be significantly modified during treatments with a constant dose. In many cases, they were accompanied by general malaise and profound weakness, part of which possibly explained by anemia. Stomatitis was another frequently observed side effect, usually mild and never requiring interruption or modification of the treatment. Skin rashes, jaw pains or postural hypotension or alopecia were never observed.

The percentage of the theoretical dose actually received and the median duration of treatment are given in Table 6 for each treatment schedule. At 15 mg/kg and 33 mg/kg the actual dose is close to 100% of the theoretical figure. At 40 mg/kg weekly, the actual dose, corresponding to 84% of the theoretical one, is not significantly greater than the dose received at 33 mg/kg.

DISCUSSION

The most striking result of this study has been the antitumor activity observed in adenocarcinoma of stomach and pancreas. In a previously published Phase I study of AAFC, one objective response was also observed in three patients with pancreas carcinoma [6]. As AAFC is partially transformed *in vivo* to

Table 5. AAFC: Relationships between dose, schedule and toxicity

		15mg/kg/day × 5		20mg/kg/day × 5		33mg/kg/week		40mg/kg/week	
		*	†	*	†	*	†	*	†
Median	Day 1	12.2	13.0	12.1	12.4	13.5	13.1	13.0	13.6
Hemoglobin (g/100ml)	Lowest	8.7	8.9	7.7	8.4	9.7	9.3	9.4	9.2
Time of nadir (week)		6	6	4	4	6	5	5	5
Median	Day 1	7.2	7.1	4.8	7.5	8.0	6.8	7.9	7.3
Leucocytes ($\times 10^3/mm^3$)	Lowest	2.2	1.4	1.2	0.3	3.3	3.0	3.0	3.8
Time of nadir (week)		4	4	4	4	5	4	5	5
Median	Day 1	260	245	155	244	266	279	260	215
Thrombocytes ($\times 10^3/mm^3$)	Lowest	94	36	11	13	137	147	132	121
Time of nadir (week)		3	3	4	4	5	4	4	5
Nausea and vomiting (% of patients)		70%	75%	100%	75%	71%	88%	64%	85%
Mucositis (% of patients)		22%	25%	0%	0%	11%	20%	14%	0%

*Prior treatment.
†No prior treatment.

Table 6. Fraction of theoretical dose received

Dose (mg/kg) and schedule		Dose received theoretical dose
15 (×5d)	All	94%
	Pretreated	94%
	Not pretreated	94%
20 (×5d)	All	97%
	Pretreated	100%
	Not pretreated	90%
33 (w.)	All	93%
	Pretreated	94%
	Nor pretreated	92%
40 (w.)	All	84%
	Pretreated	85%
	Not pretreated	81%

ara-FU. it could be questioned whether the observed antitumor activity in digestive adenocarcinomas must be related to FU-like effect. There are serious arguments against this hypothesis. The fraction of AAFC converted to ara-FU is very small [1]. Moreover, the absence of activity of AAFC in colorectal tumors and of difference in response for patients previously treated or not is hardly compatible with AAFC acting as an analog of FU.

AAFC seems thus more probably to act as an analog of ara-C, either after transformation in ara-FC, or as AAFC itself. The antitumor effect of ara-C in gastric and pancreatic carcinoma has not yet been completely demonstrated, although tumor responses have been reported [9–11].

Epidermoid carcinoma of head and neck is the only other tumor where a low grade activity has been documented. This finding is in concordance with observations made in Phase I study, where two responses have been noticed for the same tumor type [6].

Comparing the two treatment schedules used here, the first observation is that the majority of responses have been obtained after weekly i.v. injections. Given the limited number of responses obtained no clear conclusion can be drawn from this fact, except that AAFC is active when injected once weekly thus allowing for long term out-patient treatment. The total dose tolerated in 3 or 6 weeks is smaller when AAFC is given in 5 days every 3 weeks than once weekly. At 15 mg/kg/day, in regimen 1, the total dose in 6 weeks is 150 mg for a toxicity close to the upper tolerable limit. At 33 mg/kg weekly in regimen 2, the total dose in 6 weeks is 200 mg with a lower toxicity than in regimen 1. This schedule dependence of tolerable dose resembles that observed with other pyrimidine nucleotide analogs like ara-C [9] or 5-fluoro-2′-deoxyuridine [12].

In conclusion AAFC can be considered as a new depot form of ara-C with increased resistance to deamination. It can be safely and efficiently used by single weekly rapid intravenous injections during 3–6 weeks or even more. The foregoing data suggest that AAFC is active in various solid tumor types, particularly adenocarcinoma of the stomach and pancreas. The results of this early Phase II study obtained on a limited number of patients support the view that the antitumor activity of AAFC deserves a further clinical investigation, particularly for solid tumor types where ara-C has shown some activity.

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Letter to the Editor

New E.O.R.T.C. Trial on Early Hodgkin's Disease (HV)

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ONE OF a number of working parties and cooperative groups of the European Organisation for Research on Treatment of Cancer (EORTC), is the Radiotherapy–Chemotherapy group.

Under the presidencies of Professor Tubiana, Professor Breur, Professor van der Werf-Messing and Professor Abbatucci, this group is conducting a number of clinical trials on early stages of Hodgkin's disease (H I, H II), on non Hodgkin lymphomas (L-R I, L-R II), on Ewing sarcoma (E I) and on osteosarcoma (O II).

The early results on the H I trial have been published [1]. Reports on the other trials are being prepared and will be submitted to the *European Journal of Cancer* [3]. New trials, on Hodgkin's disease stage III B, osteosarcoma and ovarian tumors are in progress of formulation.

A new trial on early stages of Hodgkin's disease (clinical stage I and II above diaphragm) (H V), has started in January 1977. The final protocol was decided by Burgers (Amsterdam), Hayat (Paris), Lebourgeois (Paris), Rosenburg (Stanford), Sizoo (Rotterdam) and Tubiana (Paris). The aim of this trial is to compare intensive initial treatment with a high chance of cure but certain risks with less intensive initial treatment, and in case of relapse, second treatment with curative intent. The patient material will be stratified into two groups, one with favourable prognostic signs, the other with less favourable prognostic signs. The subgroup with favourable prognostic signs includes patients who have LP or NS histology and stage I, or stage II with positive mediastinum and age below 40 yr and have ESR below 70 mm and a negative spleen.

This group comprises about 25% of the patient material and for them splenectomy is obligatory. The treatment comparison is between mantlefield irradiation, 4000 rad in 4 weeks, and mantlefield + paraaortic bar irradiation, 4000 rad in twice 4 weeks.

The remaining patients have less favourable prognostic signs. Splenectomy is not done in these patients. Their treatment consists of either total nodal and spleen irradiation, 4000 rad in 2 or 3 periods of 4 weeks, or 3 courses of MOPP followed by mantlefield irradiation to 3500 rad in 4 weeks followed by 3 further courses of MOPP. The endpoints to be studied are:

(a) Length of disease free survival in first, and second remission, and survival.
(b) Complication rate (including sterility), and performance status.
(c) Development of secondary malignant disease.

Some of the reasons for setting up this trial are as follows:

1. In the H I study (mantlefield irradiation only, no splenectomy), in the favourable group the disease free survival is 72 % (mean FU 8 yr). In the H II study (mantlefield + paraaortic bar irradiation and negative splenectomy), the disease free survival is 94% (mean FU $2\frac{1}{2}$ yr). As a number of relapsing patients in this part of the H I study could be cured with a second treatment, there may be some overtreatment in this H II subgroup.
2. In the H II study, there is no difference in disease free survival with splenectomy or spleen irradiation. No kidney damage has as yet come to light. In the patients with less favourable prognostic signs, and NS histology, where no chemotherapy was added, the recurrence rate was high (50%) mainly in iliac glands or in irradiated areas.
3. According to a British lymphoma study [2] for patients in stage IIIA (pathological), total nodal irradiation gave a higher

remission rate than MOPP (6 courses), namely 70% vs 50% (mean FU 2 yr). Recently it was proposed to combine MOPP with "iceberg" irradiation.

4. MOPP induces sterility in males and some females. Total nodal irradiation induces no sterility in males. Females below 40 yr are rare in the "less favourable" group. The ovaries can be transposed but it is also permitted to omit iliac irradiation in this group. Fewer secondary malignancies are expected in the TNI group than in the MOPP + RT group (from several as yet unpublished sources: ± 1%).

We expect the following rates of disease free survival in the respective groups:

(a) Favourable signs, mantlefield irradiation only: >70%. High chance of cure in relapsing patients with second treatment.

(b) Favourable signs, mantlefield + paraaortic bar irradiation: 95%.

(c) Less favourable signs, total nodal irradiation: >70%. Second treatment (eg MOPP) possible in relapsing patients.

(d) Less favourable signs, MOPP + RT: >70%.

European Centres desiring more information, or desirous of participation in this trial, are invited to write to the secretariat of the EORTC Radiotherapy–Chemotherapy Group.

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Letter to the Editor

Preliminary Trial of 9-Hydroxy-2-Methyl Ellipticinium (NSC 264–137) in Advanced Human Cancers

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ELLIPTICINE and some of its derivatives are endowed with antitumor properties in animal [1–3]. 9-Methoxy-ellipticine has been used with some success in human myeloblastic leukemia [4–5]. The effects of these compounds could be related to their ability to bind and intercalate into DNA [6]. According to this hypothesis, a search for new ellipticines was carried out with an *in vitro* screen. Before being tested in standard conditions on experimental tumors, the compounds were first assayed for their affinity to DNA. Along these lines, we have selected a new compound, 9-hydroxy-2-methyl ellipticinium (9H2ME in this paper), carrying a quaternarized nitrogen, which displays a very favorable chemotherapeutic index on leukemia L 1210 [7]. This drug is submitted to a clinical trial on solid human tumors: the preliminary results concerning the first 18 patients are reported in this paper.

All the patients had advanced solid cancers which could not be treated longer with other usual chemical drugs, either owing to the fact that they were inefficacious or as a consequence of a severe impairment of the leucocyte or platelet blood count.

Table 1. *Preliminary results obtained with 9-hydroxy-2-methyl ellipticinium in* 18 *advanced cancers*

Number of patients	18 (14 ♀; 4 ♂)
Weekly doses	from 4 mg/m^2 to 110 mg/m^2
Number of injections in each patient	from 2 to 26
Total dose in each patient	from 10 to 1800 mg
Number of remissions	8: breast 5 (bone metastases—local recurrences) bronchus 1 (small cell anapl.) thyroid 1 (anaplastic) colon 1 (lung metastases)
Duration of effect	from 1.5 month to > 7 months
Failures	8 (7 breasts—1 larynx)
Not evaluable	2

The drug was administered intravenously at weekly intervals. The first patients were given 4 mg/m^2 in each injection as this weekly dose seemed to be reasonable according to previous toxicological studies on animals [7]. Yet in the face of the unexpected good tolerance of 9H2ME by patients, these injections were progressively increased up to 110 mg/m^2. As a matter of fact, one did not usually exceed 70–80 mg/m^2 as digestive troubles occurred more frequently and intensively beyond this dose.

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The results of this trial are summarized in Table 1. In this table, "objective remission" is defined as an obvious objective clinical, radiological or scintigraphic improvement lasting longer than 1 month, whatever the importance of this improvement.

In the 16 patients who could be evaluated, administration of 9H2ME resulted in objective remissions whose length at the present time reaches from 1 month to > 7 months.

Leucocytes and platelets counts were non affected by this treatment even in patients who were given high doses. No enzymatic trouble, no renal disturbance were found. In the matter of side effects, one has only noted nausea or vomiting for a short time in about half of the patients, transient variation of blood pressure (increase in 3 cases, decrease in 2 cases) and transient rise of temperature (6 cases). The only serious accident which was noted concerns an anaplastic carcinoma of the thyroid. Paradoxically this accident underlines the efficiency of 9H2ME. The patient's lesions which surrounded the neck shrank rapidly. Feeding *per os* could be resumed. The patient died suddenly from a jugular-carotid haemorrhage as a result of the tumor regression.

These preliminary data demonstrate that a new ellipticine derivative, characterized by a low hematologic, hepatic, nervous or renal toxicity, is able to induce objective remissions in solid human cancers, mainly breast tumors and its metastases. It is submitted to further clinical studies in order to determine the optimal indications, dosages and schedules.

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Announcement

E.O.R.T.C. SYMPOSIUM ON CONTROVERSIES IN CANCER TREATMENT

To be held in Brussels on 26–29 April, 1978, the first day of which will be devoted to controversies on design and analysis of clinical trials.

Abstracts (in English) of free communications on cancer therapy must be submitted by 15 February, 1978 (to be presented on 29 April).

For details and registration, write to: Dr. M. Staquet, E.O.R.T.C. Data Center, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Bruxelles.

This symposium will be held 2 rue Evers, 1000 Bruxelles (1st floor). Attendance will be limited to the first 200 registrations. Registration fee: 3000 Belgian francs.

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Recent Journal Contents (1978)

International Journal of Cancer — *January, 1978*

Human Cancer

E. Beth, M. Cikes and G. Giraldo: Microfluorometric analysis of anticomplement and indirect immunofluorescence tests for human papova-virus (JCV and BKV) T antigens.

S. A. Sherwin, R. E. Benveniste and G. J. Todaro: Complement-mediated lysis of type C viruses: effect of primate and human sera on various retroviruses.

K. Tabuchi, W. M. Kirsch, M. Low, D. Gaskin and S. Maa: Screening of human brain tumors for SV40-related T antigen.

D. C. G. Skegg: BCG vaccination and the incidence of lymphomas and leukaemia.

H. Y. Cho, J. S. Rhim and P. Arnstein: Chemical transformation of human revertant cells induced by murine sarcoma virus.

K. Nooter, J. Overdevest, R. Dubbes, G. Koch, P. Bentvelzen, C. Zurcher, J. Coolen and J. Calafat: Type C oncovirus isolate from human leukemic bone marrow: further *in vitro* and *in vivo* characterization.

O. F. Eremin, J. Ashby and J. P. Stephens: Human natural cytotoxicity in the blood and lymphoid organs of healthy donors and patients with malignant disease.

O. F. Eremin, R. R. A. Coombs, D. Plumb and J. Ashby: Characterization of the human natural killer (NK) cell in blood and lymphoid organs.

Experimental Cancer

N. Kuzumaki, E. M. Fenyö, B. C. Giovanella and G. Klein: Augmented immunogenicity of low antigenic rat tumors after superinfection with endogenous murine C-type virus in nude mice.

I. Gresser, C. Maury, M.-T. Bandu, M. Tovey and M.-T. Maunoury: Role of endogenous interferon in the antitumor effect of poly I·C and statolon as demonstrated by the use of anti-mouse interferon serum.

T. Ishibashi, H. Yamada, S. Harada, Y. Harada, M. Takamoto and K. Sugiyama: Inhibition and promotion of tumor growth by BCG: evidence for stimulation of humoral enhancing factors by BCG.

N. Takeichi, C. W. Boone, H. T. Holden and R. B. Herberman: Immunological study of two stocks of Moloney sarcoma virus producing regressor and progressor tumors in C57BL/6 mice.

G. Carbone, G. Invernizzi, A. Meschini and G. Parmiani: *In vitro* and *in vivo* expression of original and foreign H-2 antigens and of the tumor-associated transplantation antigen of a murine fibrosarcoma.

J. J. Collins, F. Sanfilippo, T.-C. Lynn, R. Ishizaki and R. S. Metzgar: Immunotherapy of murine leukemia. I. Protection against Friend leukemia virus-induced disease by passive serum therapy.

G. Y. Gillespie and S. W. Russell: Development and persistence of cytolytic T lymphocytes in regressing or progressing Moloney sarcomas.

B. W. Calnek, K. K. Murthy and K. A. Schat: Establishment of Marek's disease lymphoblastoid cell lines from transplantable versus primary lymphomas.

K. Nelson and H. O. Sjögren: Effects of presensitization *in vivo* on cell-mediated responses to embryonic antigens *in vitro*.

M. Haas: Genetic analysis of *in vitro* leukemogenesis induced by thymus epithelial reticulum cells transmitting murine leukemia viruses.

G. G. Hatch, P. J. Balwierz, B. C. Casto and J. A. DiPaolo: Characteristics of hamster cells transformed by the combined action of chemical and virus.

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Papers to be Published

M. Bistrović, B. Nagy, Ž. Marčić and K. Kolarić

Interaction of adriamycin and radiation in combined treatment on mouse L-cells.

L. Olsson, P. Ebbesen, N. Kiger, I. Florentin and G. Mathé

The antileukemic effect of systemic non-specific BCG-immunostimulation vs systemic specific immunostimulation with irradiated isogeneic leukemic cells.

K. D. Chandradasa and R. M. R. Barnes

The correlation of the ongoing proliferative response to tumor in lymphoid organs with the anti-tumor activity of the lymphoid cells.

M. Cikes

Expression of hormone receptors in cancer cells: a hypothesis.

M. Fiorani, R. Butler, L. Bertolini and R. Revoltella

Early events during C1300 neuroblastoma cell interaction with syngeneic lymphocytes.

P. Falk

Patterns of vasculature in two pairs of related fibrosarcomas in the rat and their relation to tumour responses to single large doses of radiation.

E. M. Uyeki, T. Nishimura, T. N. Miller and T. U. Bisel

Effect of methotrexate and aminopterin on tritiated nucleoside suicide of colony forming cells, *in vitro*.

M. D. Foadi, A. M. Slater and G. D. Pegrum

Kinetic features of a lymphoid population in the blastic crisis of chronic granulocytic leukaemia.

J. R. W. Masters, R. A. Hawkins, K. Sangster, W. Hawkins, I. I. Smith, A. A. Shivas, M. M. Roberts and A. P. M. Forrest

Oestrogen receptors, cellularity, elastosis and menstrual status in human breast cancer.

P. Whur, C. M. Urquhart, D. C. Williams, D. Donaldson and A. Sakula

Fibrinolysis in lung cancer.

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Perspectives in Cancer Research

Expression of Hormone Receptors in Cancer Cells: A Hypothesis

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Abstract—*It is postulated that the quantitative and qualitative changes of hormone receptors observed in cancer cells are a consequence of retrogenic expression resulting in appearance of cellular properties characteristic of certain developmental stages of the normal parental cells. As an example, the high levels of hormone receptors found in some human breast cancers may reflect a transition to normal developmental stages of the mammary gland. The occurrence of "ectopic" hormone receptors in cancer cells may be regarded as a paradigm of "ectopic" hormone production observed in a variety of human tumors. The altered expression of hormone receptors may be responsible for excessive hormonal stimulation of cancer cells.*

INTRODUCTION

MALIGNANT transformation of cells is not infrequently accompanied by quantitative and qualitative changes in responsiveness to hormonal stimulation. Since hormones exert their effects on target tissues via specific receptors located either on the cell surface or in the cytoplasm, it seems probable that the changes in hormone responsiveness in neoplasia are a result of variations in the number of functionally active hormone receptors.

The hypothesis advanced here postulates that the changes of hormone receptors in cancer cells reflect the transition of cellular properties to certain developmental stages of their normal counterparts.

Conceptually, the idea of reversion in cancer cells to more immature developmental stages can be traced back to 1874 and since then it has been revived in various forms [1]. The occurrence in cancer cells of fetal enzymes and antigens is well documented (for review see ref. [2]). For the purpose of this discussion, it is of secondary importance whether the transition to more immature stages has been affected by blockade of the normal ontogenetic process or by dedifferentiation of normal adult fully differentiated cells. However, it is clear that neoplastic tissues frequently demonstrate properties characteristic of fetal cells and tissues.

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QUANTITATIVE CHANGES OF HORMONE RECEPTORS IN CANCER CELLS

The best known example of quantitative changes of hormone receptors is human breast cancer. The determination of hormone receptors in human breast tumors has been widely used to predict tumor response to hormonal treatment [3]. These studies have revealed a wide range of estrogen receptor (ER) content in extracts from breast cancer tissues [4, 5]. Although the ratio of parenchymal to connective tissue components can account for some of the many fold differences in the concentration of ER found in the malignant breast tissues, it is insufficient to explain the wide range [6]. Moreover, normal resting mammary gland tissue, a target organ for estrogen, contains comparatively low or undetectable amounts of ER [7–10].

The levels of ER found in some human breast cancers are comparable to those found in the mammary gland during lactation. It is interesting to mention that as early as 1896 Sir George Thomas Beatson, who inaugurated ovariectomy for palliation of advanced human breast cancer, noticed a similarity between breast cancer in young women and the lactating mammary gland. "It was clear to me that the changes that take place in the mammary gland in the process of lactation are almost identical, to a certain point, with what takes place in a cancerous mamma [11]."

In agreement with Beatson's original observation are the more recent findings showing a similarity in hormone receptor content in breast tumors and the lactating mammary gland. Beers and Wittliff [12] found that ER content in infiltrating ductal carcinoma of human breast was similar to that of the lactating mammary gland. Prolactin receptor content in R3230AC rat mammary carcinoma was similar to that of the normal rat lactating mammary gland [13], while the levels of prolactin receptors in DMBA-induced rat mammary tumors may even exceed that in lactating mammary gland [14]. addition, Hilf *et al.* [15] showed that transplantable rat mammary carcinoma R3230AC responded to estrogen with enzyme changes associated with lactation. The same rat mammary tumor had similar RNA-ase and RNA-ase inhibitor levels as the lactating rat mammary gland [16].

Alterations of hormonal responsiveness in neoplastic tissues have frequently been observed in animal models. Rat mammary tumors induced by dimethylaminobiphenyl had about 5 times higher levels of cyclic AMP and responded stronger to epinephrin stimulation than normal breast tissue [17]. It seems probable that the responsiveness of tumor tissues to hormonal stimulation, as measured by changes in adenyl cyclase activity reflects, at least in part, the quantitative changes of specific hormone receptors. Machia *et al.* [18] and Mandato *et al.* [19] showed that the reduced activation of the adenyl cyclase activity in a transplantable rat hepatoma after stimulation with TSH was due to a decrease in binding of the hormone to the tumor plasma membranes. Prostaglandin caused a greater augmentation of cyclic AMP in malignant hepatomas than in uninvolved liver and benign hyperplastic nodules of ethionine treated rats [20]. An increase of the isoprotenol responsiveness and the decrease of the glucagon responsiveness was observed in the preneoplastic livers of rats fed a diet containing 3'methyl-4-dimethyl-aminoazobenzene [21]. These changes in hormone responsiveness were similar to those found in fetal and perinatal rat liver [22, 23]. A comparison of cyclic AMP response to glucagon stimulation in a series of Morris hepatomas with various degrees of differentiation showed that the response of highly differentiated hepatomas was similar to mature rat liver, whereas the response of the less differentiated tumors resembled that of fetal or newborn rat liver [24]. Kelly *et al.* [25] reported that the increase of cyclic AMP after stimulation with catecholamines and prostaglandins was much higher in SV40-transformed WI-38 cells than in the normal untransformed cells. Mammary tumors induced by estrogen and progesterone in spayed GR mice had ten times higher levels of estrogen receptor than normal mammary gland tissues from the same animals [26].

These findings suggest that the alterations of responsiveness to hormonal stimulation and the changes of the levels of hormone receptors observed in tumorous tissues might reflect a transition in tumor cells to certain developmental stages of the normal fully differentiated parental tissues.

QUALITATIVE CHANGES OF HORMONE RESPONSIVENESS IN CANCER CELLS

The first indication of tumor stimulation by "inappropriate" hormones was obtained in a corticosterone producing rat adrenortical carcinoma [27]. ACTH stimulated the adenyl cyclase activity both in the tumor and normal rat adrenal tissue. However, the tumor adenyl cyclase activity was stimulated by epinephrine, norepinephrine and thyroid-stimulating hormone (TSH) while the normal rat adrenal tissue was unresponsive to these hormones. The tumor possessed also receptors for luteinizing hormone (LH) and follicle stimulating hormone (FSH) as well as the beta adrenergic receptor. The tumor enzyme failed to respond to glucagon, vasopressin, parathyroid hormone or thyrocalcitonin. The same authors later examined a series of human tumors for the response to hormone stimulation [28]. Two parathyroid adenomas composed of chief cells were stimulated by glucagon while an oxyphil parathyroid adenoma failed to respond to any hormone tested. These investigators did not examine normal human parathyroid tissue for stimulation with glucagon. However, the cyclase activity of dog parathyroid has been reported to respond to glucagon stimulation [29]. Schorr *et al.* [28] found that the cyclase activity of a thyroid follicular carcinoma was stimulated by TSH and LH. The TSH stimulation was more intensive in the tumor than in the colloid goiter. However, the posssibility of contamination of LH preparations with TSH was not excluded. ACTH stimulated the adenyl cyclase activity of a chromophobe hypophyseal adenoma. Normal dog pituitary was not stimulated with ACTH. A pheochromocytoma from a patient with bilateral localization of the tumor responded to glucagon, ACTH and TSH stimulation. Pheochromocytomas from another two patients were unresponsive to any hormone tested. The cyclase activity of the normal bovine adrenal medulla

was reported to be unresponsive to glucagon [30]. In a later study Hinshaw and Ney [31] reported that 3 of 9 human pheochromocytomas were stimulated by glucagon while the normal human adrenal medulla did not respond to this hormone. Two of four human adrenocortical tumors were responsive to both ACTH and LH and one of them responded with a 40% decrease in 17-hydroxy-steroid excretion after administration of triiodothyronine in doses sufficient to suppress the endogenous TSH. The other two adrenocortical tumors were not stimulated with any of the hormones tested.

More recently, Lacombe *et al.* [32] observed a qualitative change of adrenoreceptors in Zajdela hepatoma of rat. Normal rat liver possesses the β_2 adrenoreceptor characterized by stimulation with catecholamines in the following order of potency: proctokylol > isoproterenol > adrenalin > noradrenalin. The adrenoreceptor found in Zajdela hepatoma was of the β_1 type with catecholamines in the following order lamine stimulation: isoproterenol > proctokylol > noradrenalin > adrenalin.

EXPRESSION OF HORMONE RECEPTORS AND GROWTH CONTROL

Cellular changes relevant to malignant behaviour of cells will be those that will enable the cells to survive and multiply in an environment that imposes an efficient growth control on normal tissue components. Generally, 2 ways can be envisaged of how cells could escape the growth control signals. One is the acquisition by the cells of receptors for growth promoting stimuli; the other is a loss of receptors responsible for mediation of growth restricting influences. The hypothesis advanced here is primarily concerned with the former escape mechanism. The high levels of hormone receptors frequently found in cancer cells is a prerequisite for the stimulating effect of circulating hormones on cancer cells. The concentrations of hormone receptors in a proportion of human and animal tumors were higher than in the normal parental tissues and comparable to that of the parental tissues during the fetal stage or the physiologic periods characterized by high susceptibility to hormonal influences. These findings make it likely that the quantitative and qualitative changes observed in cancer cells are a consequence of the acquisition by cancer cells of hormone receptor properties characteristic of stages in the normal differentiation of the cell of origin.

It is conceivable that the presence in neoplastic cells of high amounts of hormone receptors will enable these cells to proliferate in an environment with relatively low concentration of circulating hormones, while normal cells will be less stimulated in the same environment. A significant correlation has indeed been found between the degree of prolactin binding activity in rat mammary tumors and prolactin-induced growth response of these tumors [33]. Consistent with these observations is a report demonstrating a direct correlation between ER concentration in human breast tumors and their response to hormonal treatment [4].

The appearance in cancer cells of "ectopic" hormone receptors promotes autonomy of the tumor since the tumor cells may be stimulated by hormones other than the ones which stimulate the normal parental tissue. "Ectopic" hormone receptors may be regarded as a paradigm of ectopic hormone production, a phenomenon frequently encountered in a variety of human tumors [34]. The production of ectopic hormones by tumor tissues is not a chaotic process; it is regarded as an ontogenic phase-specific event whose appearance is determined by the embryological origin of the normal parental tissue [34]. The same might apply for "ectopic" hormone receptors.

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Early Events During C1300 Neuroblastoma Cell Interaction with Syngeneic Lymphocytes

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Abstract—*Rosette formation leading to specific target cell lysis has been shown between spleen lymphocytes and syngeneic cloned C1300 neuroblastoma cells. These two phases of cell interaction could be separated by low temperature, EDTA or cytochalasin-B, which permitted the formation of rosettes but inhibited lysis. Significant differences were also seen between the various clones of neuroblastoma tested. Additon of EDTA or cytochalasin-B shortly after rosette formation prevented target cell lysis, but addition later than* 5–10 min *from rosette formation was ineffective, indicating a triggering of lysis and/or critical damage shortly after cell adherence.*

INTRODUCTION

EVIDENCE has been recently provided that autogenous immunity to C1300 neuroblastoma (NB) cells exists in syngeneic A/J mice [1]. Spleen cells of unsensitized mice or mice with a growing NB tumor recognize antigenic differences on the membrane surface of syngeneic NB cells in a one way mixed lymphocyte-tumor blastogenic assay system [2] or by chromium release from prelabeled NB target cells [3]. Morphologically, the first event of such cell recognition reactions consisted of adherence of lymphocytes to NB cells [4]. The purpose of this study was the characterization and analysis of the early phases which follow first cell contact and lead to target cell lysis.

Cell adherence was documented by an *in vitro* rosette formation between attacking lymphocytes and NB cells. After binding lymphocytes were observed for a potential cytotoxic effect on target cells. This was measured by dye-exclusion or chromium release assays.

MATERIAL AND METHODS

1. *Reagents*

The following drugs were used: cytochalasin-B (I.C.I. Research Lab., Cheshire, England), colchicine, (Sigma Chemical Co., St. Louis, Mo), vinblastine (Velbe, Eli Lilly and Co., Indianapolis, Ind.). All solutions of drugs were freshly prepared and used immediately. Ethylen-di-amino-tetracetic acid (EDTA) was from Sigma.

2. *C1300 neuroblastoma (NB) cells*

Cloned neuroblastoma cells (Clone NB1R, NB6R, NB9R and NB10R) were isolated in our laboratory from a C1300 NB line originally supplied 5 yr ago by Dr. F. Jacob (Institut Pasteur, Paris). These cells are maintained in suspension culture as anaplastic cells in 20% Fetal Bovine Serum (FBS) in Dulbecco's modified Eagle's medium in 12% CO_2 and air atmosphere. When the cells are allowed to adhere to tissue culture vessels in the absence or in low concentration of FBS, they transform, rapidly emit cytoplasmic processes and acquire several biochemical and morphological characteristics of mature neurons [5]. Evidence has been recently provided that different clones derived from the same C1300 NB cell line share a common set of tumor-associated antigenic determinants which are mainly responsible for tumor cell recognition by syngeneic lymphocytes *in vitro*. A different quantitative expression of such antigenic determinants was seen, however, among the clones and in respect to the original line. Clone NB6R appeared to be the most stimulatory for syngeneic spleen cells and exhi-

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bited high expression of such common NB-associated antigen(s) [2].

3. *Mice*

Normal 2 month male A/J mice (The Jackson Laboratories, Bar Harbor, Maine) were inoculated sub-cutaneously with 2×10^6 NB6R per mouse and spleens were dissected 7 days thereafter, at a time when the growing tumor mass at the site of cell injection was 2 × 2 mm in size.

4. *Lymphocyte suspension*

Lymphocytes were obtained from spleens by the Metrizoate–Ficoll method (Lymphoprep, Nyegaard and Co, AS, Oslo) [6]. Defibrinated non adherent-spleen cell preparations were centrifuged at room temperature (300 × ***g*** for 30 min) and the white cells at the interface between medium and Lymphoprep consisted mainly of lymphocytes (over 90%), monocytes and few granulocytes (1–2%).

5. *Rosette technique*

NB cells in suspension culture were washed twice and resuspended before the test in serum-free Earle's balanced salt solution (EBSS) to a concentration of 10^5 viable cells ml^{-1}. Five hundred microliters of spleen lymphoid cells were mixed with 0.1 ml of neuroblastoma cells at various cell ratios in round-bottomed plastic test tubes, centrifuged (300 ***g***) for 5 min at room temperature and gently resuspended. The cells were examined for rosette formation in a haemocytometer and Giemsa stained cytocentrifuge preparations, using a Shandon Elliott Cytospin cytocentrifuge. In some time course kinetic studies of rosette formation, cells in pellet were resuspended in their own medium and immediately fixed in equal vol of 0.1% paraformaldehyde in the same diluent to "freeze" the reaction at each desired time. NB cells with four or more lymphocytes attached were counted as rosettes. Rosettes were kept at desired temperature and examined for cell viability. Aldehyde (2.0% paraformaldehyde, 2.0% glutaraldehyde, v/v 1:1) fixed rosettes were also prepared for electron microscopy [7].

6. *Assay for cell cytotoxicity*

Labeled neuroblastoma cells [8] (5×10^4 ^{51}Cr) were pipetted into round-bottom glass tubes (100 × 7.5). Varying concentrations of spleen lymphocytes were added at a lymphocyte to target (L/T) ratios ranging from 2:1 to 200:1 in a final vol of 0.5 ml RPMI-1640 medium containing 2% heat decomplemented and filtered FBS. The cells were centrifuged (300 ***g***) for 5 min. The pellets were then gently resuspended before incubation at 37°C in 5% CO_2 in 95% air. After 1 hr the tubes were centrifuged and 0.5 ml of medium were aspirated for gamma counting. Per cent specific cytotoxicity was expressed as:

$$\frac{\begin{array}{l}{}^{51}Cr \text{ release in experimental} - \\ {}^{51}Cr \text{ release in controls (NB cells alone)}\end{array}}{\begin{array}{l}\text{maximum } {}^{51}Cr \text{ release } (+5\% \text{ SDS}) - \\ {}^{51}Cr \text{ release in controls (NB cells alone)}\end{array}} \times 100$$

Target cell killing *in vitro* was also demonstrated by the erythrosin-B method after resuspending the cells in their own medium.

7. *Inhibitors*

Tumor membrane antigens. A soluble, crude cell extract was prepared by stirring $2–5 \times 10^8$ NB6R cells for 16 hr at 4°C in 3 M KCl, pH 7.4 Tris-buffered saline followed by dialysis and high speed centrifugation (105,000 ***g*** for 2 hr at 4°C). This cell product, named crude soluble antigen (CSA), was shown to be immunogenic when added *in vitro* to syngeneic spleen cells from unprimed A/J mice; it also induced a significantly increased incorporation of ^3H-Th by spleen lymphoid cells from neuroblastoma bearing mice inoculated 7 days before with 2×10^6 viable tumor cells [2]. A second tumor cell extract was obtained by a mild papain digestion of crude membrane preparations of NB6R cells followed by Sephadex G-150 fractionation. A soluble product, named crude membrane antigen (CMA, mol. wt below 30,000) was recovered in peak III as elsewhere described [2]. CMA was a very effective and specific inhibitor of blastogenesis of syngeneic A/J spleen lymphoid cells against NB cells in a unidirectional mixed cell culture system. On the contrary, it proved to be poor stimulatory when challenged *in vitro* with lymphocytes from normal A/J mice or from mice bearing neuroblastoma tumours. Soluble cell products from spleen, liver and kidney cells of normal A/J mice were prepared in a similar manner and were used as a source of negative controls.

Antibodies. The 7S fractions from 2 rabbits injected subcutaneously 15 days previously with 2×10^7 aldehyde fixed (0.07% formaldehyde and 0.02% glutaraldehyde) NB6R cells were obtained by recycling Sephadex-G-200 and DE-52 (Whatman) column chromatography (0.005 M ionic strength pH 7.8 phosphate buffer) and was free of active complement or 19S antibody. This globulin fraction was then absorbed on spleen, liver and kidney of normal

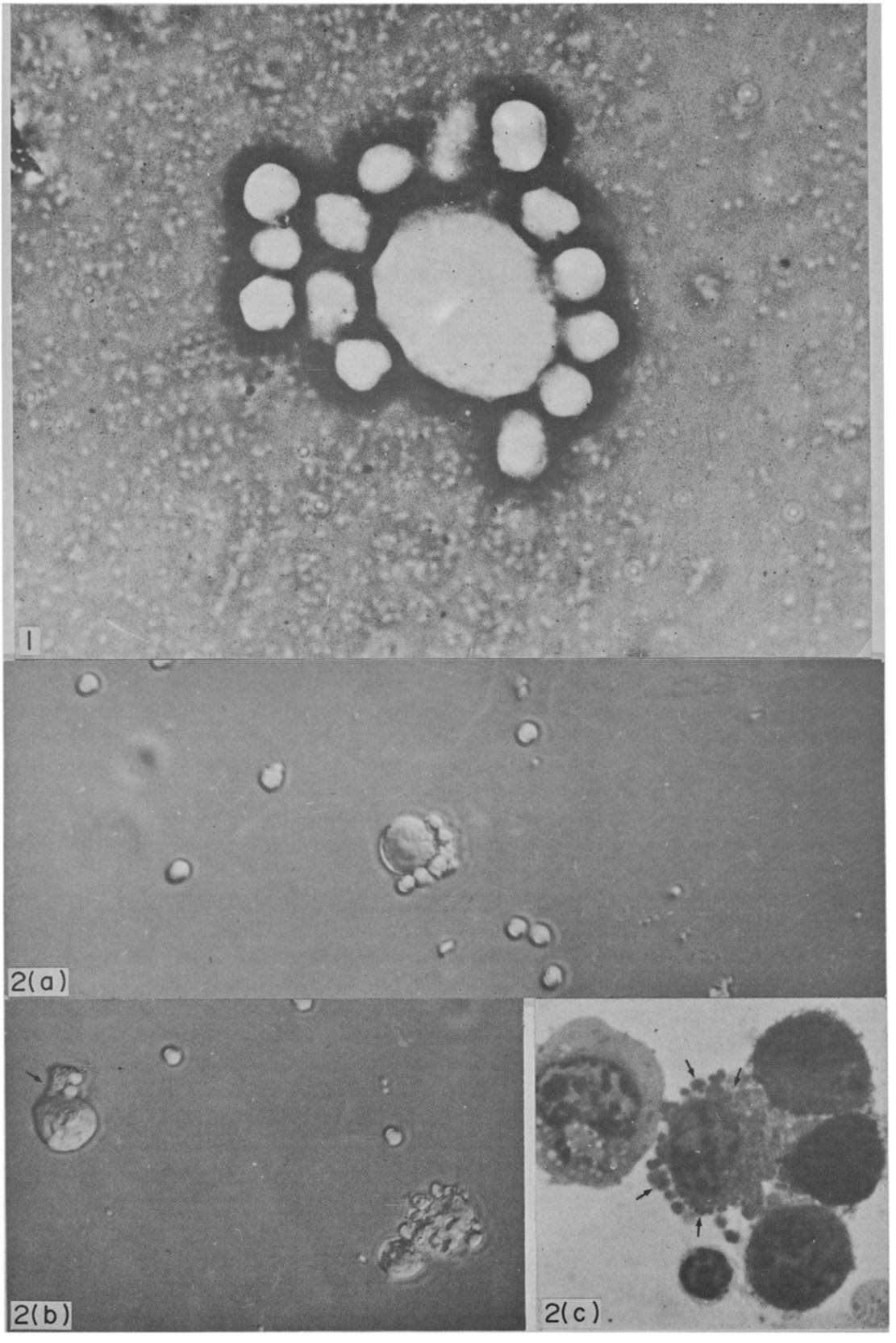

Fig. 1. *Attacking spleen lymphocytes from neuroblastoma bearing A/J mice form a rosette around syngeneic NB6R cells* (*Phase contrast, dark ground,* × 2400).

Fig. 2. *Lymphocytes from neuroblastoma bearing A/J mice, forming rosettes around syngeneic NB6R cells.* (*a*) *Wet preparation after* 15 min *at* 37°C (*phase contrast,* × 800). *Note a preferential binding of lymphocytes to a polar region on the surface of the target cell.* (*b*) *Arrow indicates cytoplasmic blebs departing from the target cell in close vicinity of bound lymphocytes.* (*c*) *Cytoplasmic blebs in a damaged rosette forming NB6R cell* (*semifine preparation,* × 2000).

A/J mice. In the presence of guinea pig complement (1:10) this 7S fraction was effective in killing *in vitro* 100% NB6R cells at 1:30 dilution after 60 min at 37°C. In the absence of added complement no cell killing was obtained. The 7S immunoglobulin fraction from a rabbit hyperimmune serum against human serum albumin absorbed on normal A/J cells was used as a source of negative control. Fab preparations of 7S immunoglobulins were obtained by papain digestion [9]. Anti-theta serum was prepared by giving C3H mice a course of seven injections of A/J thymus cells, at weekly intervals. All the sera were pooled and absorbed on normal liver and kidney A/J cells. The cytotoxic effect of anti-theta serum was tested on A/J mouse spleen cells by a chromium release assay or the erythrosin-B dye method: more than 70% of normal spleen lymphocytes were killed by this antiserum at a dilution of 1:10 in the presence of complement. Sepharose 4B beads (Bio-Rad, Richmond. Cal.) were covalently coated with rabbit anti mouse gamma globulin 7S immunoglobulins (MGG) as described by Wigzell *et al.* [10]; by sensitive radioimmunoassay, using ^{125}I-labeled 7S-MGG [11], it was calculated that 1 μg of beads (dry weight) bound at saturation about 10^7 molecules of ^{125}I-MGG.

RESULTS

1. *Rosette technique*

Firm adherence of syngeneic spleen lymphocytes to C1300 neuroblastoma cells in suspension was demonstrated by the rosette technique. Lymphocytes bound to NB target cells at sites distributed at random on their surface (Fig. 1). When, however, the cells in the pellet were left to incubate for longer than 5–10 min at 24°C or higher temperature, there was a tendency for lymphocytes (usually less than 10 per target cell) to bind at a restricted polar area on the target cell and blebs from the cytoplasm of NB cells rapidly formed firstly in the proximity of the areas of contact of adhering lymphocytes and then over the whole cell surface (Fig. 2).

Increasing numbers of dead NB cells were then counted; dead cells did not form rosettes. When different NB clones were compared for rosette-forming capacity with lymphocytes, all clones were capable of forming rosettes. However, significant differences were shown among the clones. For example, cells of clone NB10R, even when they were incubated with a large number of lymphocytes, formed few rosettes (but many cells with 1–3 lymphocytes attached). In contrast, the majority of cells from clone NB1R and NB6R formed rosettes with usually 10–20 lymphocytes bound per cell, even if they were exposed to few lymphocytes in the test (Fig. 3). Using T-lymphocytes enriched spleen cell population (after filtration through Sepharose-beads covalently coated with anti-mouse immunoglobulins antibody) almost 100% NB6R cells formed rosettes with 10–20 lymphocytes firmly bound per cell (lymphocyte to target cell ratio 32:1). Under the same experimental conditions, using a B-lymphocyte enriched cell population (after treatment with anti-theta antibody and 1:10 fresh guinea pig serum as a source of complement, at 37°C for 60 min) few (5–6%) rosettes were obtained with the majority of NB6R cells binding a maximum of 1–2 lymphocytes per cell. Preincubation of NB cells with anti-NB6R Fab-antibody consistently inhibited rosette formation. Cell adherence was prevented by pre-exposing A/J lymphocytes to soluble NB cell extracts (CSA) or to a solubilized crude membrane antigen (CMA) from NB6R cells before exposure to NB cells. If similarly made preparations from normal A/J mouse cells or cell membrane were tested, no inhibition of rosette formation was obtained. These results are reported in Table 1.

2. *Target cell lysis*

After binding, the lymphocytes were observed to kill rosette forming NB target cells *in vitro* in the absence of added serum complement, as demonstrated by the erythrosin-B dye method. The kinetic of target cell lysis in experiment performed at 37°C followed an S-shaped curve. Using NB6R cells, an initial lag phase of less than 10 min was seen until 50% cell lysis took place within 15 min. In the chromium release assay, the cytotoxic effect of lymphocytes on these target cells was detected after an initial lag time of less than 15 min at room temperature (less than 5 min at 37°C), increasing rapidly and reaching 50% cell lysis in less than 20 min at room temperature (in about 15 min at 37°C). Using 30 min of incubation at room temperature as a standard procedure, it was shown that maximal target cell lysis was observed at an optimal lymphocyte to target cell ratio 16–32:1. The results of a typical experiment are reported in Fig. 4. When in these experimental conditions different NB clones were compared as target cells in the chromium release assay, following rosette formation with syngeneic normal lymphocytes, significantly different ratios of lymphocytes were required to induce a maximum release of chromium by a fixed number of pre-labeled cells, with the different clones. As a general observation, clones which more easily formed rosettes

Table 1. Effects of previous treatments of attacking A/J spleen lymphocytes or syngeneic cloned (NB6R) C1300 neuroblastoma cells, on rosette formation and target cell lysis. Lymphocytes from mice inoculated 7 days before with NB6R cells, were incubated, after treatment, with ^{51}Cr labeled NB6R cells at 32:1 lymphocyte to target cell ratio. Rosette formation and ^{51}Cr release from target cells were measured as described in Material and Methods. Data are reported as mean values (±S.D.) on triplicate experiments

Treatment	Rosette formation	Specific ^{51}Cr release
Anti-theta antibody† (1:10)* plus complement	6±3	10±4
Anti-mouse γ-Globulins columns†	100	100
Anti-NB6R (Fab)‡	35±8	100
Anti-HSA (Fab)‡	100	100
Normal rabbit IgG‡	100	100
CSA (NB6R)-10 μg‡	5±2	0
CMA (NB6R)-10 μg‡	0	0
Extracts from cells or membranes of normal A/J cells	100	100

*After incubation of lymphocytes for 60 min at 37°C in the presence of 1:10 fresh guinea pig serum.
†After filtration of cells through Sepharose beads covalently coated with rabbit anti-mouse gamma globulin [10].
‡Inhibitors were added to cells 20 min before rosette formation at room temperature.

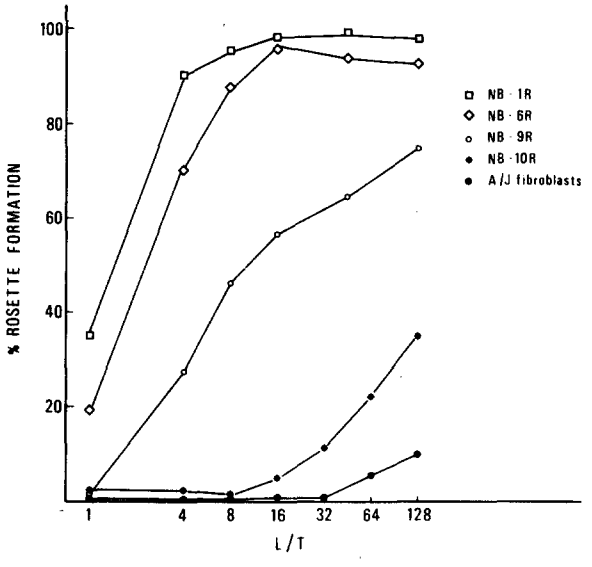

Fig. 3. Rosette formation by cloned NB cells and syngeneic lymphocytes, from mice with a growing C1300 neuroblastoma tumor at different ratios of lymphocytes to target cells. Abscissa: lymphocyte: target cell (L/T) ratio; Ordinate: per cent of NB cells forming rosettes.

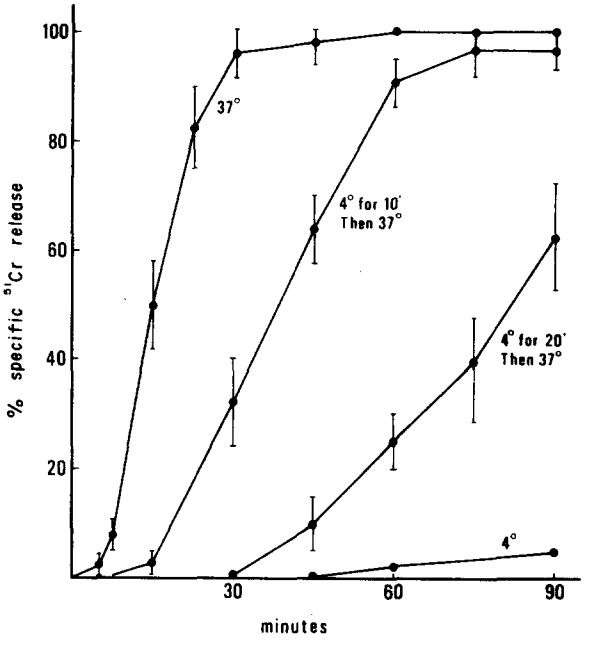

Fig. 4. Kinetic of NB6R cell lysis by syngeneic spleen lymphocytes from A/J mice with a growing C1300 neuroblastoma as detected by release of ^{51}Cr from pre-labeled target cells: Lymphocytes and target cells (32:1 ratio) were mixed at 37°C or at 4°C, then centrifuged and incubated at 37°C. Data which are reported are expressed as mean values of five experiments ± S.D. Cell-cultures which were kept at 4°C at all times, gave less than 5% cytotoxicity after 90 min.

also required a lower effector to target cell ratio for being lysed (Table 2).

CMA from NB6R cells specifically prevented chromium release from pre-labeled NB target cells. Clones which more easily formed rosettes required higher concentrations of CMA for inhibition of lysis (Fig. 5). However CMA had to be given to effector cells at an optimum interval of time before their exposure to target cells. When CMA was added to lymphocytes at the same time or after the effector cells less or no inhibition was induced (Fig. 6). Preincubation of NB target cells with Fab fragments of anti-NB6R antibody (which specifically prevented rosette formation) also inhibited cell lysis from lymphocytes. When Fab fragments were added to rosettes already formed, no inhibition of chromium release was induced. The results of these experiments are summarized in Table 1.

Table 2. Effect of different lymphocyte to target cell (L/T) ratio in ^{51}Cr *release by pre-labeled NB6R cells forming rosettes with sensitized syngeneic spleen lymphocytes. Rosette formation and* ^{51}Cr *release were measured at* 37°C *as described in Material and Methods*

Cells	Specific ^{51}Cr release (%) (L/T ratio)							
	2:1	4:1	8:1	16:1	32:1	64:1	128:1	200:1
NB	10	21	25	25	36	52	70	70
NB1R	12	25	41	65	80	92	85	90
NB6R	20	38	56	86	92	95	92	96
NB7R	5	15	12	31	35	45	65	62
NB9R	—	15	15	35	25	35	45	NT†
NB10R	—	—	10	15	25	40	45	NT†

†NT: not tested.

3. *Dissociation of tumor-lymphocyte adherence and cytotoxic phases*

(a) *Effect of temperature.* Temperature significantly affected NB cell cytotoxicity by attacking lymphocytes. Lysis of target cells did not occur at 4°C but rapidly occurred at higher temperature reaching a maximum at 37–40°C (Fig. 7). When cells were pre-cooled for 10 min at 4°C, spun to a cell pellet (350 ***g***) 5 min at 4°C), resuspended and then transferred into a water bath at 37°C, the initial lag phase was prolonged as compared to cells always maintained at high temperature and the maximal rate of lysis took place within 2–3 hr (Fig. 4). Cells pre-cooled for 20–30 min before centrifugation, formed rosettes which could be easily detached simply by pipetting the cell suspension (2–3 times). Dead NB6R cells were shown only after 1–2 hr after the temperature was raised to 37°C.

(b) *Effects of EDTA.* The adhesion of lymphocytes to rosette-forming NB cells could be reversed with EDTA (0.01 M) (Fig. 8). EDTA added shortly after cell centrifugation (less than 5 min at room temperature) separated lymphocytes from NB6R cells and target cells released significantly less chromium than did EDTA-untreated controls. If EDTA was given after 10 min from rosette formation, detachment of NB6R cells from lymphocytes was still obtained but target cell lysis was not prevented. At room temperature, separation of cells forming a rosette by EDTA took about 5–10 min to be completed: at 37°C the reaction was significantly shorter (2–3 min). In absence of EDTA, mechanical shaking or pipetting and/or low temperature were usually ineffective in separating the attached cells.

(c) *Effect of plant alkaloids and cytochalasin-B.* Colchicine and vinblastine, two drugs which induce irreversible disaggregation of microtubular proteins, had no effect in preventing rosette formation and chromium release from rosette-forming NB cells at any concentration tested which permitted cell viability (Table 3). Cytochalasin-B, a drug which has been shown to affect a variety of cellular functions involving microfilament protein organization, did not affect rosette formation but strongly inhibited NB cell lysis by attacking lymphocytes (Table 3). In the presence of cytochalasin-B, uniform binding by lymphocytes onto the target cell surface was seen, even at 37°C, without tendency of lymphocytes to gather on a polar region of the target cell.

The effect of the drug was dose dependent with an optimum at 10 μg/ml (Fig. 9). The effect was reversible since washing and replacing of medium immediately induced target cell lysis. Cytochalasin-B inhibited cell lysis if given before or immediately after (less than 5 min) cell adhesion. If given 10 min after rosette formation cytochalasin-B did not prevent target cell lysis (Fig. 9).

DISCUSSION

Our findings clearly indicate that spleen lymphocytes from A/J mice with a growing C1300 neuroblastoma tumor bind to and induce lysis of syngeneic C1300 NB cells *in vitro*. The effector cells have been identified mainly as T (thymus derived) lymphocytes and the specificity of the reaction was shown by the ability of Fab fragments of anti-NB antibodies and of solubilized NB membrane antigens to inhibit the rosette formation and cytotoxic tests. Significant differences in their ability to form rosettes and in the effector/target cell ratios necessary for optimal lysis were observed among the different clones of the same NB line. The quantity of rosette formation also varied significantly among the

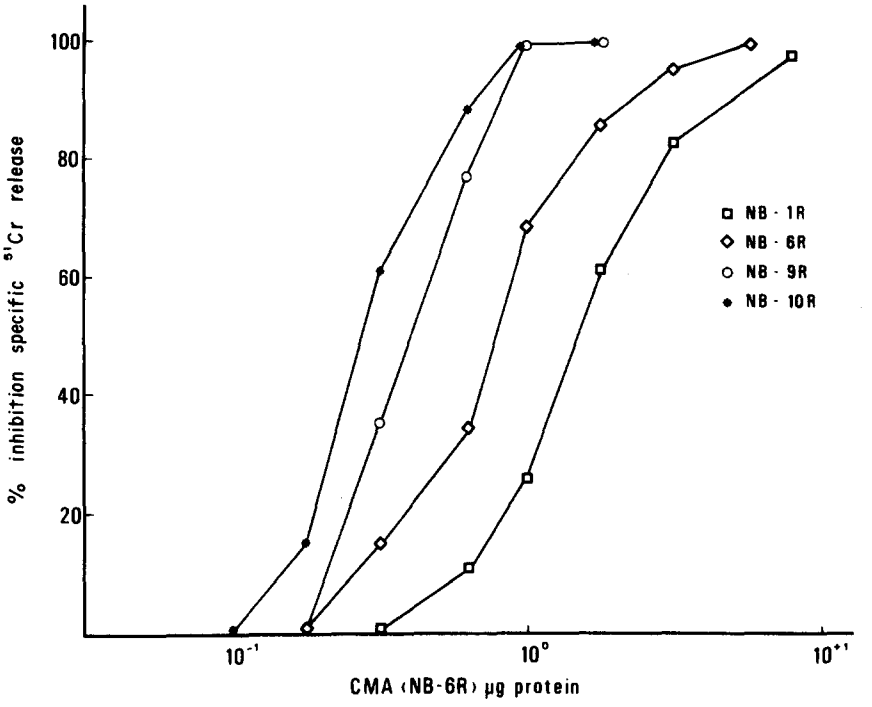

Fig. 5. Inhibition of cytotoxicity by CMA from NB6R cells. Lysis of ^{51}Cr *labeled-NB cells was induced by sensitized A/J spleen lymphocytes at effector to target cell ratio of* 32:1. *CMA and NB cells were added at the same time. The values obtained from duplicate cultures varied less than* 5%.

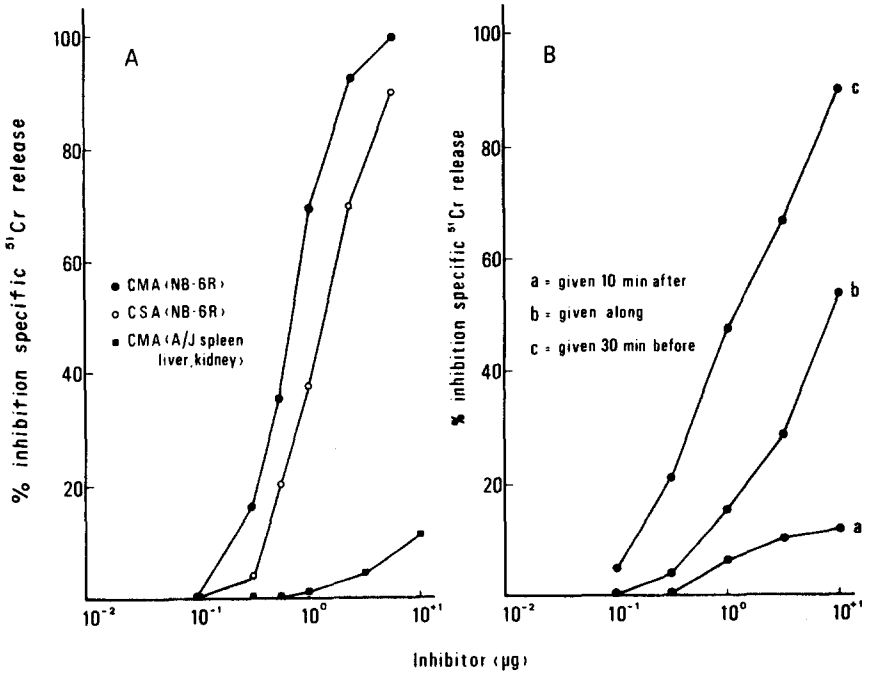

Fig. 6. Inhibition of sensitized lymphocyte mediated NB6R cell lysis by soluble crude cell extracts from NB6R cells or normal A/J spleen, liver or kidney cell extracts. Effector to target cell ratio was 32:1 *and incubation time in the cytotoxic test was* 1 hr *at* 37°C.

Panel A. Effect of total cell extract (CSA) or crude membrane extract (CMA) (see Methods). Inhibitors were added to lymphocytes 20 min *before target cells.*

Panel B. CMA from NB6R cells was added: (a) 10 min *after pre-incubation of effector and target cells; (b) along with effector and target cells; (c) target cells were added* 30 min *after pre-incubation of CMA with effector cells.*

The values obtained from triplicate cultures varied less than 5%.

Table 3. Effect of plant alkaloids and cytochalasin-B on rosette formation and target cell lysis between attacking sensitized syngeneic spleen lymphocytes and NB6R cells. Data represent mean values (± S.D.) of triplicate experiments

Drug	Dose	Lymphocyte† viability	Inhibition of rosette formation		Inhibition of ^{51}Cr release from pre-labeled NB6R cells
			4°C	37°C	
			(% of maximum)		
Colchicine	5×10^{-6}M	100	12±7	18±5	10±10
	10^{-6}M	75	26±5	31±11	35±7
	10^{-5}M	21	70±4	83±7	41±7
Vinblastine	5×10^{-6}M	100	15±3	12±2	8±3
	10^{-6}M	35	31±7	26±4	47±5
	10^{-5}M	12	85±9	91±7	77±7
Cytochalasin-B	10 μg/ml	100	12±2	6±3	96±5

†By the erythrosin-B dye exclusion test.

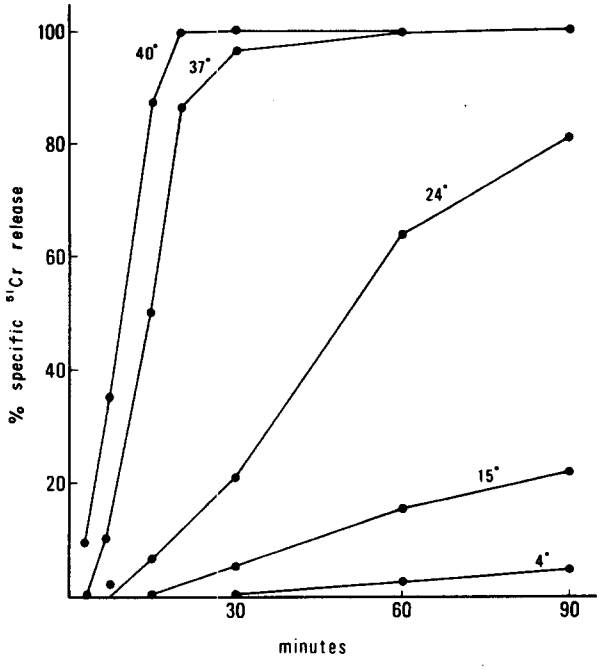

Fig. 7. Effect of temperature on target cell lysis by lymphocytes. Cultures containing sensitized A/J lymphocytes and NB6R cells were incubated at a cell ratio of 32:1 at the temperature indicated.

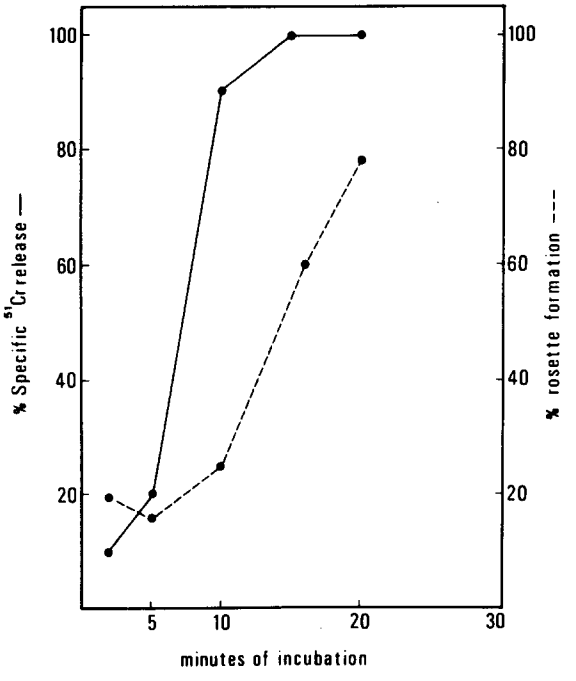

Fig. 8. Inhibition of rosette formation and cytotoxicity by EDTA. Sensitized A/J spleen lymphocytes and ^{51}Cr-labeled NB6R cells (32:1 cell ratio) were placed in culture and prepared for rosette formation. EDTA (0.01M) was added at the indicated intervals. Tubes which did not receive EDTA were used as controls. Cytotoxicity was measured after 1 hr at 37°C. The values obtained on duplicate cultures varied less than 5%.

clones (which had previously been shown [2] to vary quantitatively in the expression of NB-associated antigens), indicating a requirement for the presence and/or optimal presentation of antigenic sites on the target cells. Dead NB cells did not form rosettes, possibly due to denaturation or an altered presentation of antigenic sites.

The nature of these NB-associated antigens is as yet unknown. NB cells are known to contain cross-reacting tumor-associated transplantation antigens. Crude membrane antigens have been solubilized and some of these soluble components, at least CMA from clone NB6R, were shown not to be viral structural antigens [12]. NB cells, however, can contain RNA viral structural protein and glycoprotein antigens on their cell surface [12], and modification of histocompatibility antigens during cell transformation and differentiation *in vitro*, under conditions of cell culture in low serum concentration, has been recently observed (Invernizzi, G. and Revoltella, R., manuscript in preparation). The

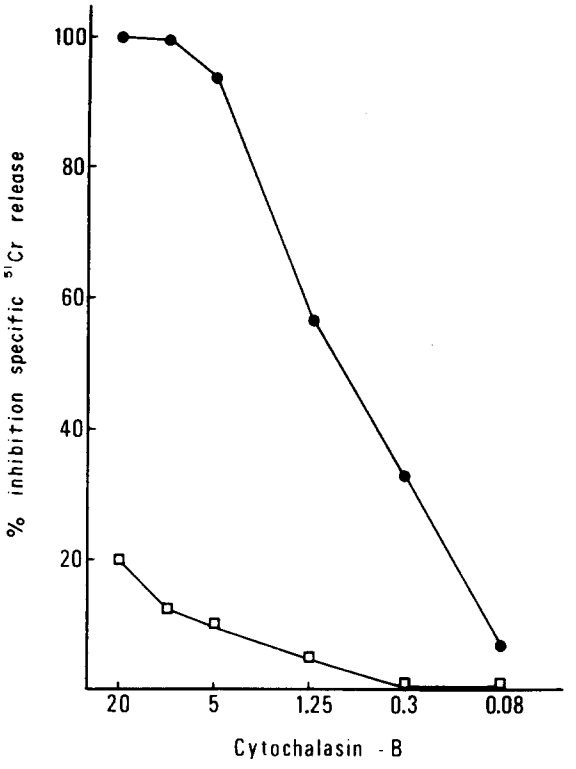

Fig. 9. Inhibition of target cell lysis by cytochalasin-B. Cultures containing sensitized A/J lymphocytes and NB6R cells were incubated at a ratio of 32:1 *at* 37°C *for* 60 min. *Cytochalasin-B was added along with effector and target cells* (●) *or,* 10 min *after rosette formation* (□).

activity of these membrane antigens has been characterized only insofar as they stimulate syngeneic lymphocytes *in vitro* [2], induce rosette formation and release ^{51}Cr in the presence of sensitized lymphocytes. The relationship between the expression of these membrane-associated tumor antigens on NB cells cultured *in vitro* to antigens of tumors growing in syngeneic recipients *in vivo* remains to be clarified.

The lytic reaction which follows rosette formation was strongly dependent on temperature, showing a sigmoidal curve with a sharp inflection at about 24°C. Rosette formation, however, was not affected quantitatively by low temperature, but rosettes formed at 4°C tended to be more fragile and to have a uniform binding of lymphocytes over the target cell surface, whereas at higher temperatures the rosettes were more stable and lymphocytes were frequently seen limited to polar regions. Cytochalasin-B showed a similar effect, i.e. inhibition of lysis but not of rosette formation and uniform lymphocyte binding even at 37°C. Cytochalasin-B has been described to interfere with the contractile microfilament system in the cell membrane [13–15] although this hypothesis is disputed since it is not clear whether the reported interference of this drug is a direct effect or results from the inhibition of other cellular functions such as phospholipid metabolism [16], oxidative phosphorylation [17], etc. Microtubular-associated functions do not seem to play a significant role in target cell lysis by lymphocytes since colchicine and vinblastine, despite being at adequate concentrations for disrupting microtubules or preventing cell growth (for review, see ref. [18]) were without effect. At concentrations at which inhibitory effects were obtained, effector cells were in fact killed by the drugs.

The inhibition of lysis by cytochalasin-B appeared to be at an early stage of the lytic process since addition after 10 min of the rosette formation reduced the degree of inhibition of target cell lysis.

CMA, which inhibited both rosette formation and target cell lysis if added initially, was ineffective at inhibiting lysis if added shortly after the beginning of the incubation.

In the presence of EDTA, the rosettes were more fragile and were easily disrupted. Significant differences were seen among the clones. When EDTA was added within 5–10 min of rosette formation, significantly less chromium release was obtained, but EDTA added later did not inhibit the cytotoxic reaction, even when the rosettes were disrupted.

These data, in conclusion, indicate that the cytotoxic reaction developed shortly after specific cell adherence. Once linkage between attacking and target cells had been established, free tumor antigen could no longer separate effector from target cells. Triggering of the cytotoxic reaction, which may involve changes in topography and/or conformation of membrane interacting sites leading to activation of the attacking cells could be prevented within 5–10 min with EDTA or by cytochalasin-B. Once triggered, the reaction became irreversible, probably because of critical damage to the target cells and lysis eventually occurred.

Recent evidence from several laboratories indicate many analogies in describing a reaction of antibody-dependent cell-mediated cytotoxicity [19, 20]. In these systems, an induced phase for lysis occurred within minutes and was followed by a lytic phase requiring several hours. These and other data involving a variey of target and effector allogeneic cell systems [21–24] all suggest at least two phases of effector mechanisms.

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Macrophage-Mediated Cytostatic Activity on Tumour Cells after Treatment with Triton WR 1339*

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Abstract—*Peritoneal macrophages collected from mice treated with schedules of Triton WR 1339 previously shown to inhibit metastasis formation in experimental tumour models were found to nonspecifically inhibit growth and DNA synthesis of tumour cells* in vitro. *No significant lysis of* ^{51}Cr-*labelled target cells could be detected. It is suggested that activation of macrophage-mediated cytostasis may contribute to the antimetastatic activity of Triton WR 1339.*

INTRODUCTION

THE NONIONIC detergent Triton WR 1339 (TWR), chemically a formaldehyde polymer of the polyoxyethylene ether of octylphenol, has been previously reported to inhibit metastasis formation in a number of animal models of cancer cell dissemination [1].

The mechanisms responsible for this anti-disseminative activity are still unclear and possibly complex, involving effects both on the cancer cell and the host [2]. In view of previous observations that treatment with this compound resulted in increased RES activity in tumour-bearing mice [3] and that TWR-treated macrophages could efficiently inhibit bacterial proliferation *in vitro* [4], it was considered of interest to investigate the possible effects of TWR on macrophage cytotoxic capacity towards tumour cells.

MATERIAL AND METHODS

Mice

Female (BALB/c × DBA2)F_1 (CD_2F_1) mice, weighing 20–22 g, obtained from Charles River, Calco, Italy, were employed throughout.

Drugs

TWR, obtained from Winthrop Co., U.S.A., was injected i.p. in 0.2 ml of 0.9% NaCl solution at a dose of 500 mg/kg for 6 consecutive days, unless otherwise stated. In most experiments, tests were performed 24 hr after the last drug injection. *Corynebacterium parvum* (*C. parvum*, lot 086P, Wellcome Research Laboratories, Beckenham, England), employed as a reference macrophage activator [5], was injected i.p. at a dose of 1.4 mg/mouse and macrophage cytotoxicity was assessed 8–12 days after this treatment.

Macrophage monolayers

Macrophage monolayers consisting of approximately 10^6 mononuclear phagocytes, were prepared as previously described [6]. Briefly, peritoneal exudate cells suspended in minimal essential medium (MEM) were seeded in the wells of Costar trays (Costar, Cambridge, Mass., USA). After 2 hr incubation at 37°C, the monolayers were washed with jets of MEM to remove nonadherent cells. Examination of Giemsa-stained preparations revealed that >95% of the adherent cells were mononuclear phagocytes.

^{3}H-*TdR uptake test*

The SL_2 lymphoma of DBA/2 origin was obtained from Dr. R. Evans, Chester Beatty Research Institute, Sutton, Surrey, England. Tumour cell DNA synthesis was evaluated as previously described [6] except that tritiated thymidine (^{3}H-TdR) was employed as a radiolabelled precursor instead of [^{125}I] Iododeoxyuridine. Briefly, 5×10^4 tumour cells were cultivated in 1 ml RPMI 1640 medium supplemented with 10% foetal bovine serum (growth medium) for 48 hr on macrophage monolayers. At the end of the incubation lymphoma cells were transferred to plastic tubes, washed twice with MEM and incubated 3 hr at

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Table 1. *Inhibition of tumour cell DNA synthesis by TWR-treated macrophages*

Macrophages	% Inhibition of ^{3}H-TdR uptake*		
	24 hr	48 hr	72 hr
Normal	7±0·5	10±1.8	−15±1
TWR	79±0.7†	73±1.2†	66±0.7†
C. parvum	82±1†	91±0.5†	75±0.4†

* Cytotoxicity was assessed after different incubation times.
†$P<0.01$ compared to normal group.

37°C with 1 μCi ^{3}H-TdR (specific activity 5Ci/mmole, Amersham, England) in 1 ml growth medium. Acid precipitable radioactivity was then determined as previously described [7] and the percentage of inhibition of ^{3}H-TdR uptake was calculated according to the formula:

$$\left(1-\frac{A}{B}\right)\times 100,$$

where A is the isotope uptake in the test group and B is the radioactivity incorporated in the presence of normal macrophages. Under these conditions normal CD_2F_1 peritoneal macrophages did not affect growth and DNA synthesis of SL_2 lymphoma cells.

^{51}Cr-*release assay*

The cytolysis assay employed in this study has previously been described [8]: 5×10^4 ^{51}Cr-labelled SL_2 lymphoma cells were incubated for different times on macrophage monolayers. Percent ^{51}Cr release was calculated as:

$$\frac{\text{isotope release in supernatant}}{\text{total releasable radioactivity per sample}}\times 100.$$

Maximal isotope release from SL_2 target cells determined by osmotic lysis averaged 75%.

Statistical analysis

Results presented are means ±S.E. of 3 replicates per experimental group. Statistical significance was assessed by Dunnet's test.

RESULTS

As shown in Fig. 1, morphologically TWR-treated peritoneal macrophages were clearly different from normal mononuclear phagocytes. In fact after 2 hr incubation at 37°C and repeated washing to remove non-adherent cells, virtually all normal macrophages were still rounded up whereas TWR treated phagocytes were completely spread out. Moreover, TWR macrophages appeared larger and markedly more vacuolated than controls. No significant differences could be detected in the number of mononuclear phagocytes recoverable from the peritoneal cavity of normal or drug-treated mice.

Table 1 shows a typical experiment in which SL_2 lymphoma DNA synthesis was evaluated after different times of *in vitro* culture in the

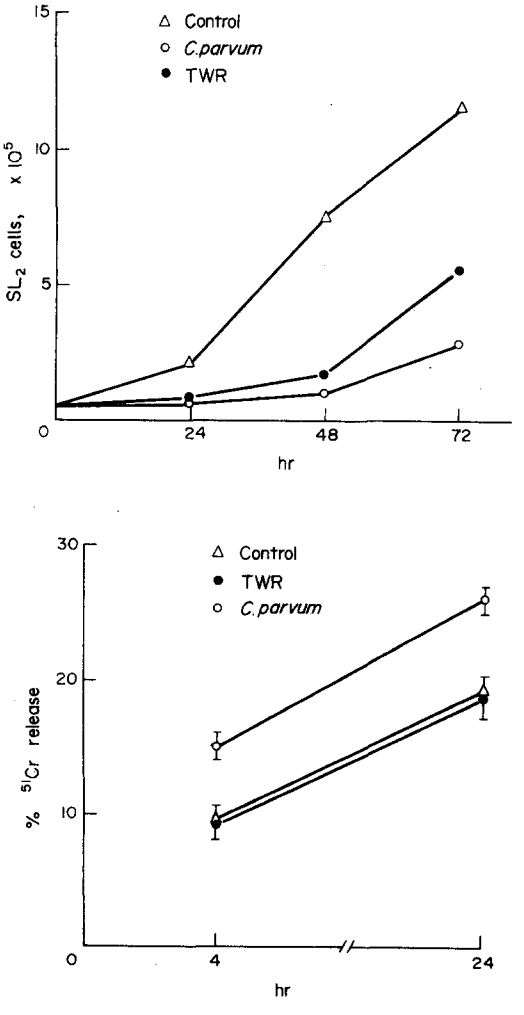

Fig. 2 In vitro *growth inhibition* (*A*) *and lysis* (*B*) *of* SL_2 *lymphoma cells by TWR or* C. parvum-*treated macrophages.*

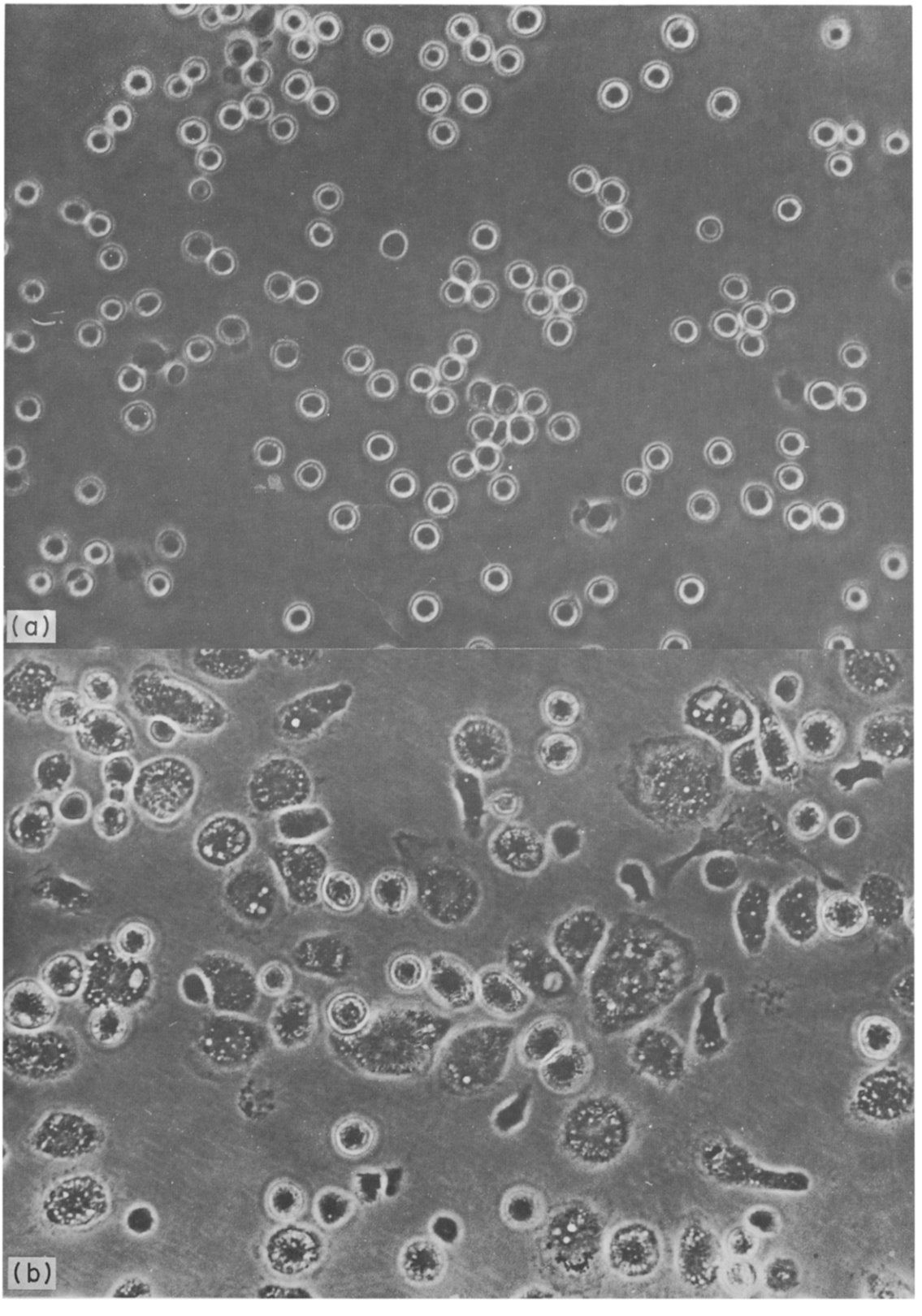

Fig. 1 *Morphology of normal* (*A*) *and TWR-treated* (*B*) *peritoneal macrophages. Phase contrast* × 128.

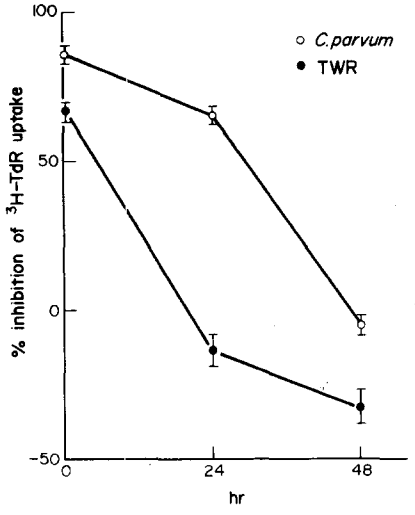

Fig. 3 Effect of in vitro *preincubation on the cytostatic activity of TWR or* C. parvum*-treated macrophages.*

presence of macrophages collected from TWR-treated animals. TWR treated mononuclear phagocytes markedly suppressed tumour cell DNA synthesis, peak inhibition being observed at 24 hr, cytotoxicity values slightly declining thereafter. Similar results were obtained when the TLX9 lymphoma or the FS6 fibrosarcoma of C57Bl origin were employed as targets (unpublished observations). Table 1 also shows that *C. parvum* treated monolayers caused greater inhibition of ^{3}H-TdR incorporation by tumour cells than macrophages collected from TWR-injected animals.

It has been shown that tumour cell DNA synthesis assays similar to that employed in these studies present a number of pitfalls [9]. Therefore in a series of experiments, macrophage cytotoxicity was evaluated either by visual counting of the number of lymphoma cells or by ^{51}Cr-release assay. As illustrated in Fig. 2, in the presence of TWR-treated monolayers significant inhibition of target cell proliferation was observed whereas no lysis of tumour cells occurred over a 24 hr observation period. Macrophages activated by *C. parvum*, employed as a reference compound in these studies, also inhibited tumour cell proliferation and in addition caused slight (4–8%) though significant lysis of target cells (Fig. 2).

In vitro cultivation of peritoneal macrophages in growth medium for different times prior to challenge with lymphoma cells resulted in rapid loss of the cytostatic activity of TWR-treated monolayers, significant stimulation of tumour cell DNA synthesis (33%) being observed after 48 hr *in vitro* (Fig. 3).

Cytotoxity disappeared similarly with *C. parvum*-treated macrophage preparations but the loss of cytostasis occurred significantly later (48 hr) than in TWR treated-monolayers (24 hr).

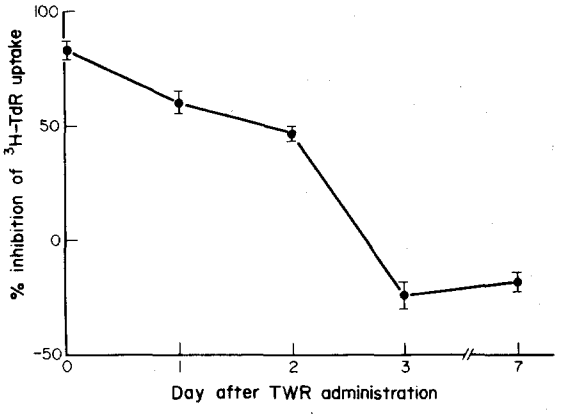

Fig. 4. Time course of the growth inhibitory capacity of TWR-treated macrophages. Macrophages were obtained from the peritoneal cavity at different times after interruption of drug administration (TWR 500 mg/kg *i.p.* × 6 days).

Table 2. Macrophage activation by TWR: effect of different drug schedules

TWR (mg/kg)	% Inhibition of ^{3}H-TdR uptake
None	2
500 × 12 days	75*
500 × 6 days	68*
500 × 2 days	−15
500	−20
250 × 12 days	52*
50 × 6 days	25

CD_2F_1 mice were treated with TWR i.p., and macrophage cytotoxicity was assessed 24 hr after the last drug injection.
* $P<0.01$ compared to controls.

A similar decay of cytostatic capacity in TWR-treated macrophages was observed *in vivo* after interruption of drug administration (Fig. 4). In fact as early as 3 days after stopping treatment with this agent, no significant phagocyte-mediated cytostasis was observed.

In a series of experiments the capacity of different TWR schedules to activate macrophages was examined.

As shown in Table 2, while significant cytotoxicity was detected following injections of 500 mg/kg for at least 6 days or of 250 mg/kg for 12 days, lower drug doses or shorter treatments resulted in a complete lack of cytotoxicity.

DISCUSSION

The results presented demonstrate that TWR administered *in vivo* renders murine macrophages cytotoxic for tumour cells *in vitro*. Cytotoxicity

under these conditions appears to be the expression of a cytostatic reaction and does not involve lysis of target cells. Thus TWR joins the now relatively long list of compounds, such as endotoxin, double stranded RNA, *C. parvum*, etc. which have been proved able to activate macrophages (see for review ref. [10]).

The mechanism by which TWR renders macrophages cytotoxic is not clear. The tuberculostatic activity of TWR-treated macrophages has been attributed to the drug interacting with lysosomes [11]. Since morphological studies have indicated that lysosomes are involved in the cytotoxic activity displayed by activated macrophages towards tumour cells [12, 13], it is tempting to speculate that phagocyte-mediated cytostasis induced by TWR may be related to this agent having some effect on lysosomes. Evidence of various types is now available to support the concept that mononuclear phagocytes play an important role in the regulation of metastatic spread [14–16]. Thus macrophage activation by TWR might contribute to the antimetastatic activity of this drug. This conclusion is supported by the observation that TWR treatments capable of activating macrophages (e.g. 500 mg/kg administered for 6 days or 250 mg/kg for 12 days, see Table 2) were previously reported to inhibit cancer cell dissemination, whereas drug schedules which did not render mononuclear phagocytes cytotoxic (e.g. 50 mg/kg for 6 days), had no effect on metastatizing tumour models [1–3].

However other pharmacological properties, such as interference with tumour cell adhesiveness, could play a role in giving this compound its antidisseminative activity [2].

The cytotoxic capacity of TWR-treated macrophages is rapidly lost following interruption of drug treatment. This could account for the need for prolonged administration of high doses of the compound in order to obtain significant antimetastatic effects [1].

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Patterns of Vasculature in Two Pairs of Related Fibrosarcomas in the Rat and their Relation to Tumour Responses to Single Large Doses of Radiation

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Abstract—*Two pairs of related lines of rat fibrosarcoma, whose responses to radiation had already been the subject of much investigation, were chosen for a study of vascular patterns. The vascular pattern in each of these four types of tumour is described and illustrated. One member of each pair has central vascular supply and drainage, the other peripheral. In one of the latter it is suggested that the pattern of vasculature results from the pressure of cell multiplication transmitted and modified by collagen at the tumour edge and investing the radial vessels. In the other line with peripheral supply and drainage, where no such distribution of collagen occurs, the pattern seems to be connected with venous infarction.*

The patterns suggest that oxygenated blood is more evenly distributed in tumours where the supply is central than in those where it is external and this conclusion is in agreement with the radiation responses.

INTRODUCTION

MANY factors affecting the growth of tumours and their response to treatment are determined by the nutrition of their cells and hence by their blood supply. Whilst the final operative portion of the vascular system in the distribution and clearance of metabolites to and from the tissues is the capillary bed, the flow of blood through it is dependent on the size and arrangement of the larger vessels, that is the arteries and veins. It has been possible to study the arrangement of these vessels in two pairs of related lines of fibrosarcoma in rats chosen because their responses to radiation had already been intensively investigated [1–10]. All four tumours arose in the same strain of pure inbred rat (John's strain of Wistar rat), all had been maintained by transplantation within this strain and the radiation responses were investigated in tumours grown at the same site. Yet in spite of the similarity between the tumours, the responses to radiation had been found to be rather different in each, especially in relation to oxygenation. For this reason, the tumours seemed particularly suitable for the study of vascular patterns, the aim being to determine how far, if at all, the vascular pattern could explain the varying responses to radiation. Details of the characteristics of the tumours are given in Table 1.

MATERIAL AND METHODS

A detailed account of the experimental procedure has been published [11]. The tumours were implanted beneath the skin on the right flank of the abdomen where they had maximum room for expansion. When the tumours had reached the appropriate size, the rats were killed by cardiac injection of disodium edetate. Fixation for six weeks in neutral buffered formalin followed and at the end of this time the treatment varied. Some tumours were bisected and after staining the two halves were mounted in Resin 4116, produced by B.I.P. Chemicals Ltd., Warley, Worcs., U.K. This is a highly transparent polyester, which acts both as a clearing agent and mountant. Material for use with it is first dehydrated in successive ethanols and then transferred to ethyl acetate before immersion in the resin. After the addition of a catalyst the specimen is blocked in a glass mould.

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Table 1. *Data from* [8] *except where otherwise acknowledged*

	SSB1A	SSB1B	RIB_5	RIB_5C
Origin	A single spontaneous fibrosarcoma in breast tissue in a breeding rat. On transplantation into several animals, the two lines were isolated and distinguished by their different growth rates.		RIB_5 was induced in 1945 by subcutaneous injection of benzopyrene. RIB_5C was derived from RIB_5 by McNally in 1966 by culturing the cells *in vitro* over a period of 12 months, during which time the plating efficiency rose from 15 to 80%.	
Histology	Spindle-celled, the cells lying with a common orientation in small interlacing bundles with collagen bundles between	Resembles SSB1A, but has more collagen between bundles.	Originally spindle-celled, but now polygonal-celled and more anaplastic.	Polygonal-celled; marginally better differentiated than RIB_5.
Volume doubling time at 9 mm dia (days).	$1\frac{1}{2}$	$4\frac{1}{2}$	1	2
Time taken to grow from 9 to 25 mm dia (days).	11.9	30	7	12.7
Necrosis	Up to 9 mm diameter no regions of coagulative necrosis and only occasional scattered partly autolysed cells. Central necrosis appears at about 18–20 mm dia.	At first as SSB1A, but no mass necrosis at later stages.	Necrosis develops in microscopic areas at maximal distance from capillary blood vessels. At 9 mm dia these constitute about 15% of tumour volume. Between 11 and 14 mm dia, these areas coalesce to form a central necrotic mass, which then increases as the tumour grows.*	Resembles RIB_5, but at later stages only scattered islands of necrosis which remain discrete.

*Thomlinson (personal communication).

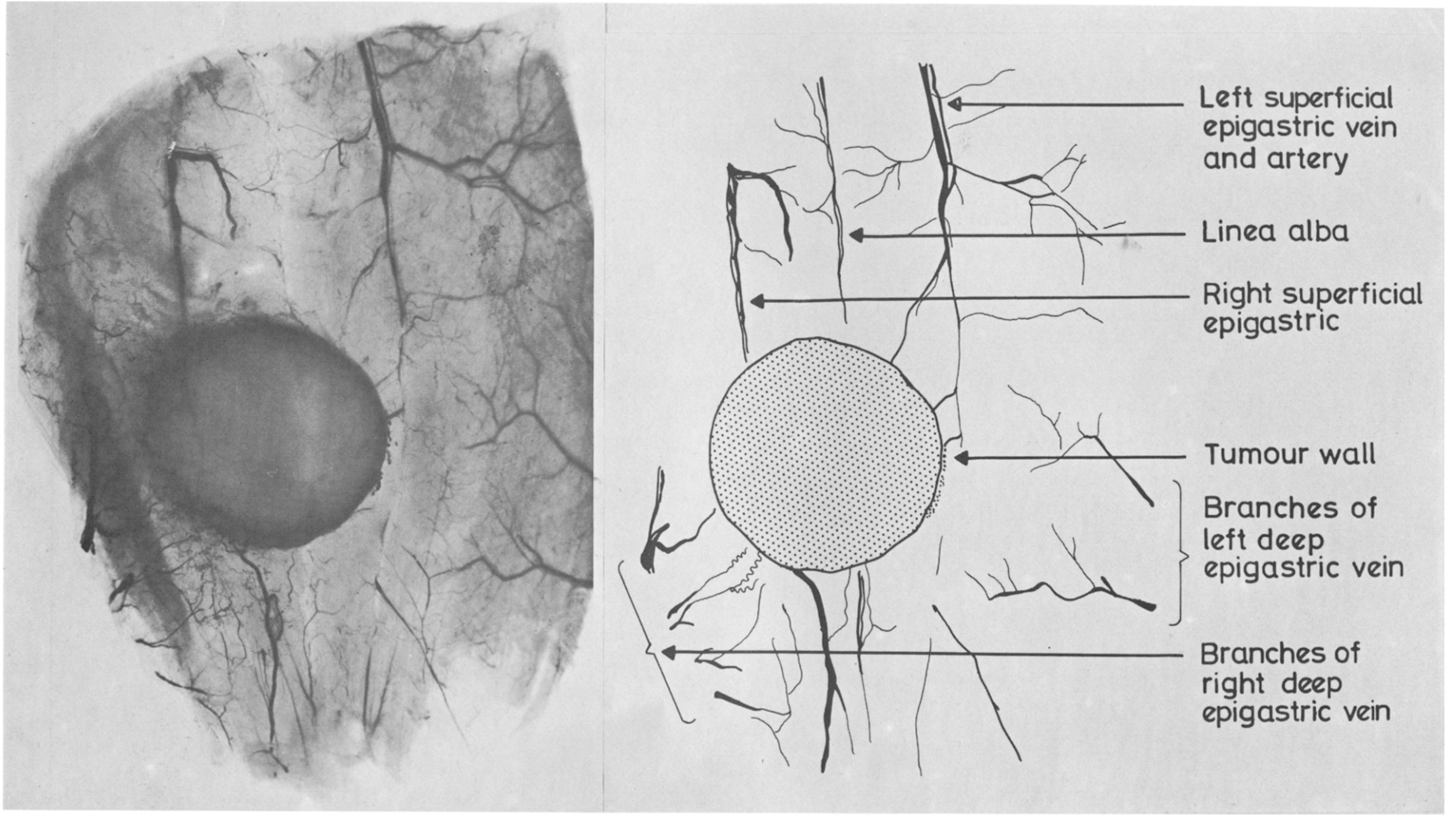


Fig. 1. *The inner half of tumour SSB1A and the surrounding abdominal wall, stained in benzidine and embedded in resin. The tumour measured* 13 × 14 × 9 mm. *The vascular supply is provided by the right superficial and deep epigastric systems with a slight contribution from the left superficial artery and vein. The centre of the tumour contains only microscopic vessels.*

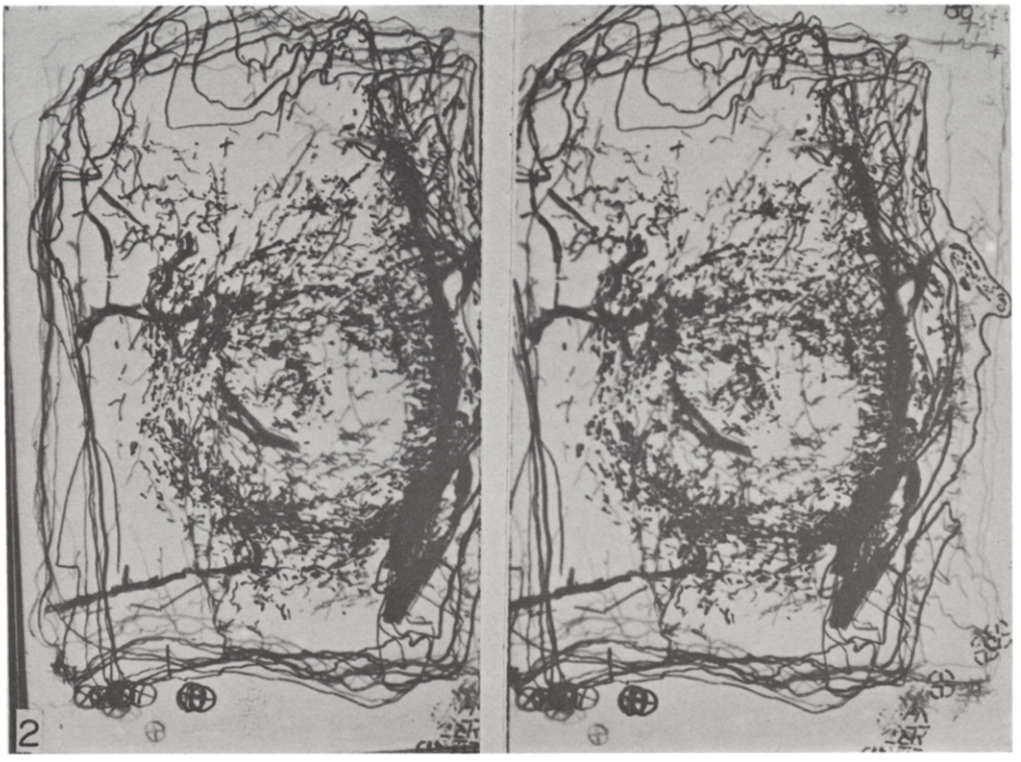


Fig. 2. Reconstruction of the vasculature of the inner half of tumour SSB1A and of the surrounding abdominal wall. The tumour measured 9.5 × 7 × 6.5 mm *and the reconstruction was made on a scale of* 20 : 1. *The wavy, superimposed lines round the edge are the outlines of successive sections. The halved tumour is hemispherical. A branch of the superficial epigastric vein lies to the right. Two small branches from the deep epigastric vein leave the tumour on the left (at* 7 *and* 10 *o'clock). The interior of the tumour is almost without blood vessels, but its walls are heavily invested. On this scale, no distinction is visible between afferent and efferent vessels. Figs.* 2, 4 *and* 5 *are stereoscopic pairs.*

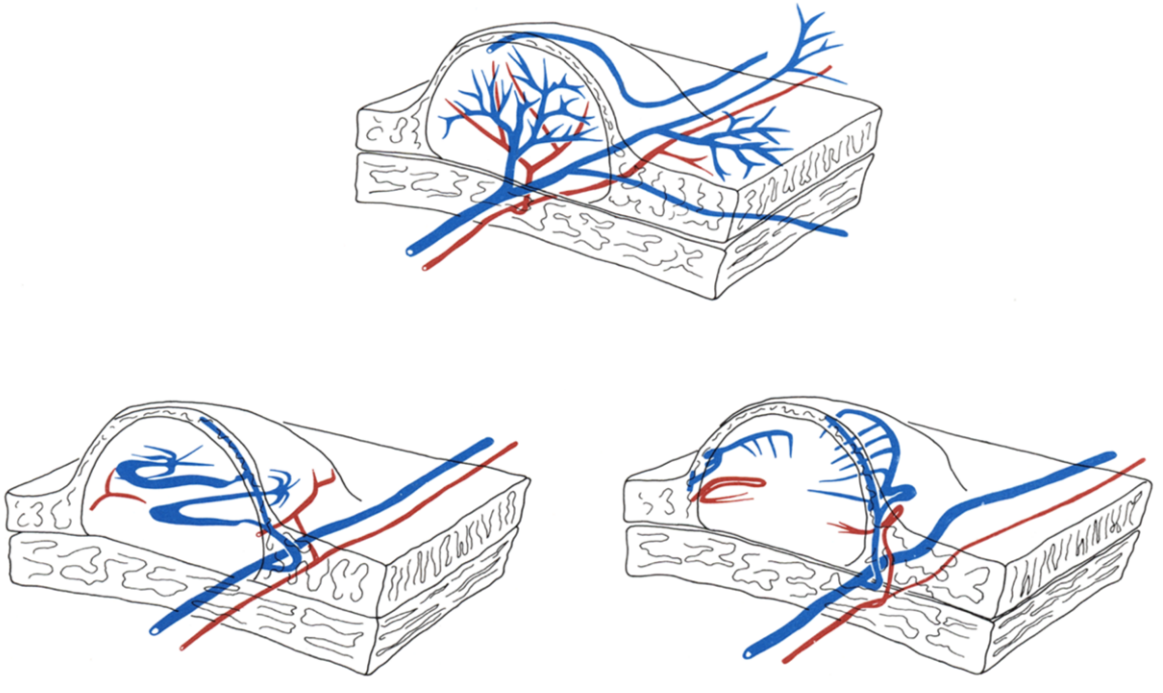


Fig. 3. The three types of pattern exhibited by the four tumour lines. Above, the internal supply and drainage pattern of SSB1B and RIB_5C; *below left, external supply and drainage exhibited by* RIB_5 *with marked constriction of the veins just before leaving the tumour and restriction of arteries to the area of venous constriction; below right, external supply and drainage exhibited by SSB1A, with highly modified veins restricted to the periphery of the tumour and arteries running over the tumour surface.*

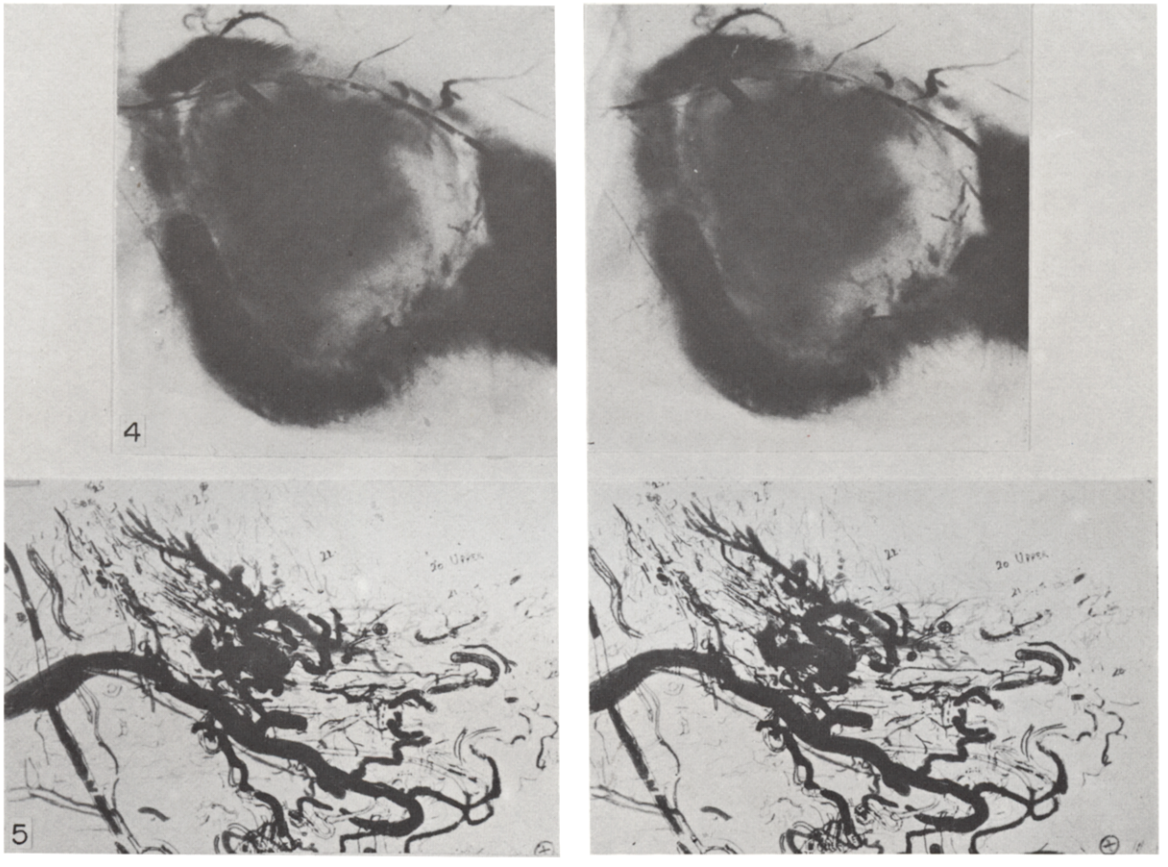
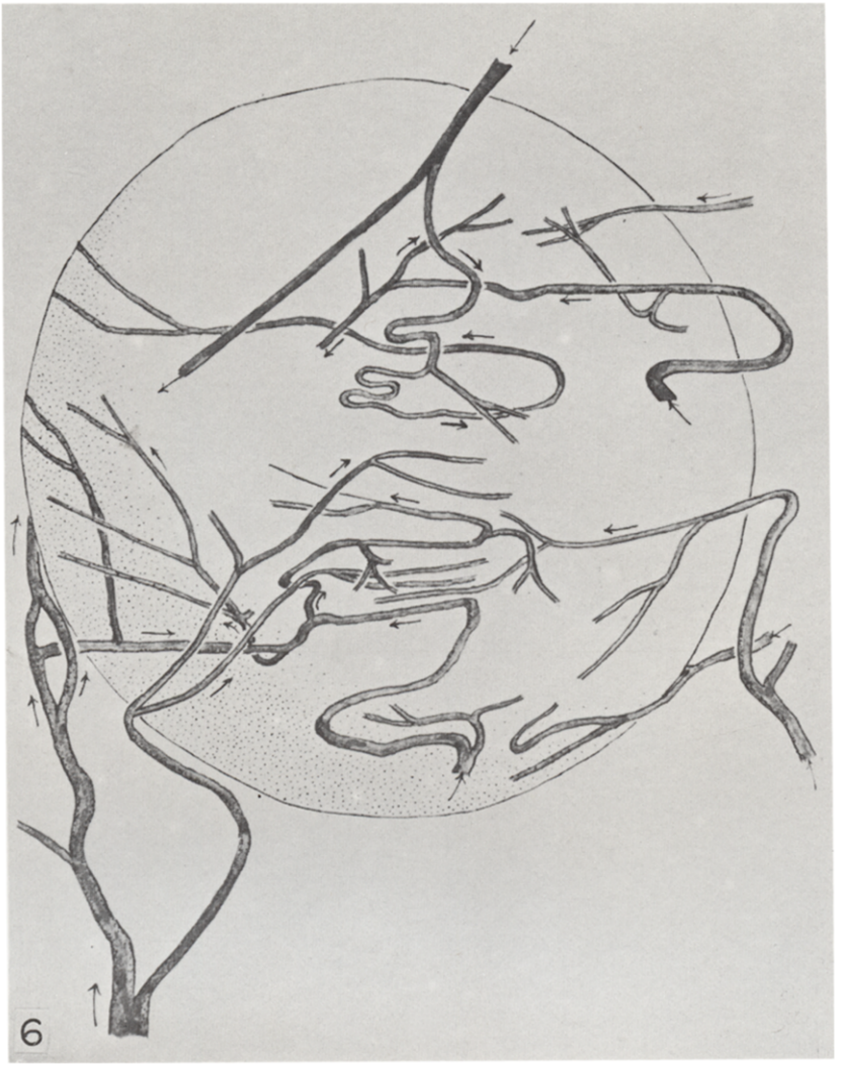


Fig. 4. The inner half of tumour SSB1B stained in benzidine and embedded in resin. The tumour is seen through the abdominal wall. The superficial epigastric vein is seen as a discontinuous line across the upper half of the picture, the vessel being constricted by the pressure of the tumour on it. The vasculature of the tumour has a shrub-like appearance and the single vein draining it can be seen entering the superficial epigastric vein. The outlines of the tumour are defined approximately by the three incidental haemorrhages which enclose it.

Fig. 5. Reconstruction of a portion of the same SSB1A tumour as in Fig. 2, the original made on a scale of × 120. *The tumour lies in the right upper portion of the photograph and the edge runs as an arc of a circle from the upper left corner to half way down the right margin. A broad vein outside the tumour ascends from the lower centre of the photograph to half way up on the left. The bases of three of its branches to the tumour are shown, but the course of the left one only is fully visible. Its base is sinuous and within the tumour it spreads circumferentially. Slightly nearer than the main vein is an artery appearing grey. An anastomosis is visible before the artery runs under the main vein (lower centre).*



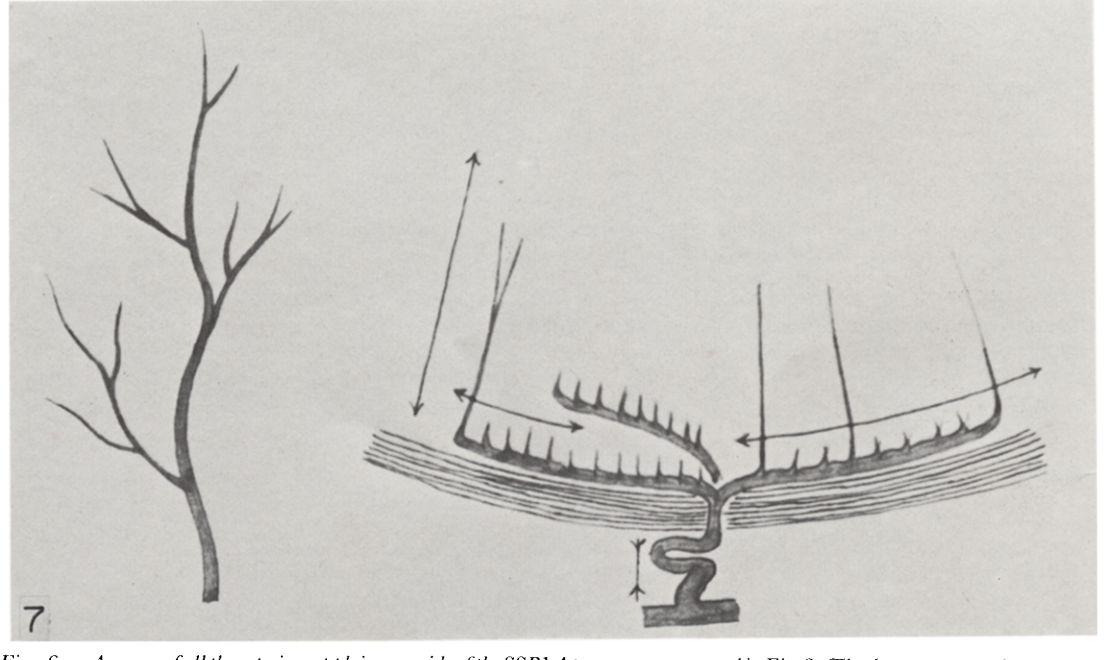


Fig. 6. *A survey of all the arteries supplying one side of the SSB1A tumour reconstructed in Fig.* 2. *The three upper arteries enter from the skin, the four lower from the abdominal wall. The arteries run over the tumour surface for a considerable distance before branching and entering it. The course of their branches within the tumour is not shown. The survey shows the uneven distribution of the arterial supply. The arrows reveal one anastomosis (lower left centre).*

Fig. 7. *A normal riverine vein on the left and the highly modified vein of tumour SSB1A on the right. Arrows represent the forces resulting from tumour growth which act on the vein. The parallel lines represent the collagen at the tumour surface. The tumour lies above these. The base of the vein is supported by loose connective tissue.*

In this method the surrounding tissues were left in place round the half tumour so that both the vascular supply to the tumour and the vessels within it were visible (Fig. 1). Tumours not treated in this way were cut with a cryostat into sections 200 μm thick. To standardize their treatment, a special technique was used for mounting the sections on glass slides. The vasculature of each section was next traced onto an acetate sheet, each sheet was supported on a pane of glass and the glass panes stacked one above another with spacers between. Placed on an X-ray viewer, the stack gave a 3-dimensional representation of the vasculature when viewed from above (Fig. 2). The blood vessels gave the alignment of adjacent sections and at a magnification of × 125 these conformed to within 2 mm.

In both methods staining was carried out by benzidine and hydrogen peroxide, which colour the blood an intense blue-black. Sections were normally counterstained with Eosin, but if it was necessary to identify collagen, Fast Green F.C.F. was used instead. The latter stain was also used for the same purpose with resin-embedded specimens. Benzidine staining was used in preference to injection methods because of its ability to reveal the state of constriction or of filling of a vessel. The methods used have the advantage that they can be employed with biopsy or post mortem material.

RESULTS

A difference that became apparent on sectioning the tumours or even when bisecting them for mounting in resin was that the sections or cut surfaces of the slow-growing member of each pair of tumours, SSB1B and RIB_5C, remained flat, whereas the sections and cut surfaces of the fast-growing members, SSB1A and RIB_5, curved in a saucer-shaped fashion. The centres of the latter thereby revealed a tendency to expand in relation to the peripheral zone. No such tendency was seen in the former tumours. For this reason the former were regarded as *lax* tumours, the latter as *tense*. Within the four tumour lines studied, this distinction coincides with a fundamental difference in the vascular patterns. This difference is illustrated in Fig. 3. In the upper figure, illustrating the vascular pattern of the two lax tumours, SSB1B and RIB_5C, an artery and a vein enter the tumour together at its base and branch together for some time. The general direction of flow of blood is therefore centrifugal in the arteries and centripetal in the veins. In contrast, in the tense tumours afferent and efferent vessels, even if they enter together, soon separate and since the main vessels are peripheral, not central, the direction of the flow of blood is centripetal in the afferent vessels and centrifugal in the efferent. For convenience throughout this paper the lax type is described as possessing central supply and drainage, while the tense type is described as having external supply and drainage. These descriptions are applicable only when the tumour is considered as an isolated unit. They are clearly inapplicable when the tumour is considered as part of the body of its host.

Although the two tense types of tumour agree in possessing external supply and drainage of blood, their patterns otherwise differ markedly (Fig. 3). In SSB1A, arteries are confined to the surface of the tumour, whereas in RIB_5 they penetrate into it, but after running not more than 200 μm turn through a right angle and continue their course just within its boundary. The form of the veins, too, is in sharp contrast, those of SSB1A running circumferentially just within the tumour before entering a main drainage vessel through a short, dilated and usually reflexed base, while those of RIB_5, which lie within the tumour and usually extend right across it, are characterized by their extreme degree of dilatation and their marked constriction immediately before leaving it.

The arrangement of the vascular supply to the tumour from the host, illustrated in Fig. 1, is in general typical of that obtaining in all four tumour lines, though the details vary both with the tumour line and with the exact point of implantation. In Fig. 1 the vascular supply comes from both the deep and superficial epigastric systems and it is noticeable that the left superficial epigastric vein and artery as well as the right contribute to the tumour's vasculature. In tumours implanted further to the rat's right, the left superficial epigastric system makes no contribution, and in the extreme case, the whole abdominal supply may come from the deep epigastrics. With tumour RIB_5, the position is similar, but for the two lax tumours, the more distant sources of supply do not occur. The kinking of two small branches of the deep epigastric vein in Fig. 1 is typical of the tense tumours and a kinking of small branches of the superficial epigastric vein may also occur in them. In the lax tumours kinking does not occur. In both tense tumours branches from the cutaneous artery and vein are present in addition to the abdominal supply.

Detailed description of the patterns of vasculature

SSB1B

Six tumours were investigated ranging in size from 14 × 11 × 10 to 24 × 19 × 14 mm.

This tumour possesses the simplest pattern of vasculature found in the four lines. An afferent and efferent vessel enter the tumour together from its abdominal side (Fig. 4) and then with frequent branching climb towards the skin. Since the afferent vessel even within the tumour retains its muscular investiture, it is clearly distinguishable from the efferent. Its branches are evenly but sparsely distributed to all parts of the tumour. The density is such that the distance between the termination of one branch and that of its nearest neighbour may be as much as 500 μm. There is no marked dilatation of the efferent vessels and the density of their fine branches is high. Intervals between their terminations seldom exceed 25 μm. No vessels filled by blood could be seen uniting the finest afferents with the finest efferents, though a faint grey network connecting the two systems could be seen in some areas. Occasional flattened erythrocytes lay on the strands of this network. A similar structure occurs in the other three tumour lines, suggesting that an extensive capillary network is present in all four tumour lines, but that it closes with the fall in arterial pressure at death. The evidence for this suggestion is considered later.

RIB_5C

Five tumours were examined ranging from 10 $\times$ 5.5 $\times$ 5 to 26 $\times$ 17 $\times$ 16 mm dia.

The vascular pattern of this tumour line resembles that of SSB1B, a vein and an artery entering the tumour together at its base and climbing together towards the skin. The artery in this case also has a well-marked muscular investiture. The pattern differs, however, in that the supply and drainage of blood afforded by these vessels is supplemented by three or four further pairs of arteries and veins which ascend the walls of the tumour and enter through its side. Within the tumour, the vessels soon cease to run side by side, but the artery retains its muscular investiture. There is no marked dilatation of the veins.

SSB1A

Fifteen tumours were investigated ranging in size from 8 $\times$ 7 $\times$ 6 to 20 $\times$ 13 $\times$ 16 mm.

The vasculature of this tumour line is strongly peripheral (Figs. 2 and 5). On reaching the tumour, arteries and veins cease to run together in pairs. The arteries run circumferentially over the surface of the tumour, many extending as much as quarter of the way round (Fig. 6). Some retrace their original course after a hairpin bend. Ultimately each divides into 2–6 branches and it is these which penetrate into the tumour. Each is only 6 μm in diameter and lacks a muscular investiture. Some penetrate as much as half way across the tumour; most end nearer the periphery. These afferent vessels tend to run straighter, to have a more uniform bore and to branch less often than efferent vessels of the same diameter, but the two can only be distinguished from each other with certainty by tracing them back to the vessel from which they were derived.

In tumours less than 9 mm in diameter, the veins as well as the arteries run circumferentially over the tumour, branching frequently with a normal riverine course. In older tumours, however, the form of their branches is highly modified (Fig. 5) and those draining the tumour acquire a characteristic pattern, though branches draining the adjacent tissues retain their riverine form. The pattern of the branch draining the tumour is most easily followed if it is traced from outside the tumour into it, i.e. against the flow of blood. Following it in this direction, the branch has a short, dilated and serpentine base which penetrates the tumour wall and immediately divides into about six arms, each running circumferentially just within the tumour. These arms are well spaced out, so that seen from outside the tumour they constitute the spokes of a wheel. There is, however, a considerable overlap between system and system. Each arm gives rise to further short branches, which again run circumferentially just within the tumour. Both the arms and their branches are dilated, but retain a muscular investiture. Along their whole length they give off efferent capillaries which run radially into the tumour.

The efferent capillaries branch very little and usually have a diameter of about 7 μm. A few are considerably broader with diameters up to 50 μm; these latter branch more often. There is a tendency for the 7 μm vessels to run in bundles, particularly in younger tumours (dia less than 10 mm). In tumours up to this size, both the coarse (50 μm diameter) and fine (7 μm diameter) vessels can be traced as much as two thirds of the way across the tumour before they disappear. In older tumours they travel for a much shorter distance before disappearing, and comparisons of vertical and horizontal sections shows that vessels running at right angles to the abdominal wall persist longer than those running parallel with it.

Staining with Fast Green F.C.F. indicates that both the 50 μm and the 7 μm efferent capillaries, though lacking a muscular coat, are invested with collagen and that the collagenous covering is continuous round the dilated circumferential

vessels and from them to a layer of collagen 5–10 fibres thick which surrounds the periphery of the tumour. Afferent capillaries are similarly invested with collagen and they too are anchored to the collagen surrounding the tumour. This distribution of collagen was not found in the other three tumour lines.

A further feature of interest seen in the two tense tumour lines, but not in the lax, is the occurrence of occasional arterial anastomoses. One occurs in Fig. 6, where it has the form of a T-junction, the two blood streams represented by the arms of the T meeting and running together down its vertical limb. Such anastomoses occur in Reinhold's sandwich preparations [12] and are of particular interest since they are starting points for vaso-constriction, which usually spreads from the junction along one horizontal limb or the other, reducing the flow along the vertical limb and eliminating the collisions that occur at the junction. Occasionally both horizontal limbs are constricted simultaneously with the cessation of flow along the vertical limb. A similar phenomenon seems to occur in the arterial anastomoses of SSB1A, since in two cases explored, the equivalent vessel to a horizontal arm of the T contained no blood, though the other two elements were well filled.

Necrosis was seen only in the largest of the SSB1A tumours. The necrotic area was almost central and measured 3 × 2 mm. No cell structure was visible and the necrosis was presumably coagulative. A ring of well filled blood vessels surrounded it extending 1–2 mm from it. These blood vessels closely resembled the efferent vessels seen leaving much younger tumours. They branched slightly more often and varied in diameter from 25 to 5 μm. They showed no connection with the vessels at the tumour's edge.

RIB$_5$

Three tumours were examined ranging in size from 10 × 9 × 7 to 12 × 9 × 8 mm.

In this tumour, as in SSB1A, supply and drainage of blood are external, but the pattern of its vasculature otherwise is quite different from that of SSB1A. After running over the surface of a tumour for a short distance, veins and arteries enter the tumour together in pairs and at once proceed on different courses. The artery, which retains its muscular investiture and branches occasionally, turns through a right angle and, though it may run quarter of the way round the tumour, neither it, nor any of its branches, departs more than 250 μm from the tumour edge. Later branches, lacking a muscular investiture, doubtless penetrate further into the tumour, but the large vessels of the efferent system make it impossible to trace them. When the veins are traced into the tumour, all but the smallest are found to constrict abruptly on entering so that for a distance of about 250 μm their channel is either bloodless and apparently closed, or at least very narrow. Further in the vessel broadens into a long, straight sinusoid, which extends more than half way across the tumour before constricting once more at its distal end. Here it is fed by a mop-like bundle of branches, and similar, but less dense, branches enter it along its sides. Towards the centre of the tumour some of these sinusoidal structures which lack a visible connection with the veins outside the tumour contain blood which seems stagnant, giving an uncharacteristic colour on staining.

DISCUSSION

(a) *The patterns of vasculature*

According to Rubin and Casarett [13], the earliest study of patterns of tumour vasculature was made just over a hundred years ago by C. Thiersch (1865) and the first investigator to work with transplantable tumours in rats was W. H. Lewis [14] whose forcible conclusion written 50 yr ago fits perfectly the four lines of tumour described here: "Each type of tumour has its own particular type of vascular pattern." Earlier in his paper he had suggested the possibility of "the continued multiplication of tumour cells exerting undue pressure on the capillaries" and his final conclusion was that "the blood vessels do not determine the growth of the tumour, but the tumour determines the growth and pattern of the vessels". Rubin and Casarett [13] were in complete agreement and stressed the importance of the stretching of vessels, as did Thomlinson [15], who pointed out the implications for radiotherapy, since stretching may lead to a hypoxic and therefore radio-resistant fraction of cells. The only modification that needs to be made to Lewis's first conclusion is that the pattern of vasculature is not static, but changes progressively with growth, each tumour having its characteristic pattern of development.

Since the publication of Lewis's paper, there have been two main lines of attack on the problem of tumour vasculature, one based on the study of fixed material, the other on living preparations. The first includes the works of: Margulis, Carlsson and McAlister [16], who examined seven tumour types in mice and showed that each has its characteristic vascular pattern, irrespective of the site of implantation, the stage of development or the strain of host;

Milne, Margulis, Noonan and Stoughton [17, 18], who showed that secondaries share the pattern of the primary neoplasm, providing that they are fully vascularized; and Rubin and Casarett [13, 19], who distinguished between internal and external supply and drainage patterns and were the first to associate vascular pattern with radiation response. The second line of investigation, employing sandwich techniques, because of the limitation on depth and because of the short life of the preparations, is not intended to disclose patterns of vasculature, but instead reveals the blood vessels alive and functioning, as well as the development of the vasculature in its early stages.

The most striking distinction between the patterns of the four lines described here is the possession by the two tense tumour lines of external supply and drainage, as opposed to the internal supply and drainage pattern of the two lax tumours. Such a distinction had already been described by Rubin and Casarett [19]. Their Murphy lymphosarcoma had central arborescent vascularisation and their Walker carcinosarcoma rich peripheral vascularisation with central necrosis. It is particularly interesting that though both tumours are fast growing, the lymphosarcoma is the faster. Extrapolated approximate volume doubling times at 10 mm diameter are: the lymphosarcoma, 24 hr, the carcinosarcoma, 36 hr. This shows that growth rate alone cannot be the factor determining the distinction in vascular pattern, since in the four types described here, the two faster have *external* and the two slower *internal* supply and drainage.

Of these two main types of pattern, internal and external supply and drainage, internal is clearly the simpler as demonstrated by the veins and arteries running together in pairs, the more normal pattern of branching of the vascular tree and the lesser degree of hypertrophy of the veins. The suggestion here is that the pattern of the tense tumours diverges from the simple type illustrated by the lax tumours for two distinct reasons. In SSB1A the pressures set up by tumour growth and the collagenous investiture of the blood vessels which anchors them to the collagen surrounding the tumour, modify the vascular pattern. In RIB_5 the pressure imposed by the actively dividing cells at the tumour's edge, seems to be responsible. Figure 7 contrasts the pattern of a normal riverine vein with that of the highly modified branch draining SSB1A. The forces on the vein resulting from the growth of the tumour are indicated by arrows. These forces are: radial stretching, resulting from increased diameter; circumferential stretching, resulting from the expanding circumference; and compression at the base of the branch, resulting from the advance of the periphery of the tumour. Providing that the vein and its branches are attached to the tumour at their proximal and distal ends and that the main vein is secured to the tumour edge, the three forces together would convert the riverine structure into approximately the pattern found in the tumour. It seems certain that the collagen which encircles the tumour and invests all the efferent vessels would inevitably provide just such an attachment and it is significant that this arrangement of collagen is found in this tumour, but not in the other three. The form of the afferent vessels may be explained in the same way. On their arrival at the tumour, they run over its surface for a considerable distance before dividing and entering it. A number turn on their original course after a hairpin bend (Fig. 6). Such courses are most easily explained by the assumption that an artery at its point of entry into the tumour, where it is anchored to the edge by collagen, is pushed back by the advancing tumour front.

In contrast, in RIB_5 there is no indication of stretching of either the afferent or the efferent vessels, nor is there any collagenous investiture of the vessels. Instead the narrowing or closure of the efferent vessels immediately within the tumour seems to indicate a compressional force operating in that zone and resulting indirectly from the pattern of the afferent vessels. Since the main branches of the afferent vessels are confined to the outermost shell of the tumour, the nutritional status of the cells in that zone is presumably maintained at a higher level than that of the cells in the centre of the tumour. Tannock [20] has shown that for a tumour with well marked tumour cords, the number of cells dividing at any time increases with higher nutritional status. In RIB_5 it is therefore reasonable to suppose that the rate of cell division is greater in the outer shell than in the centre of the tumour. In addition, according to Thomlinson (personal communication), RIB_5 causes some compression of the connective tissue round it forming a thin pseudo-capsule, which is infiltrated by tumour, so that the process advances as a wave. This pseudo-capsule would assist in maintaining the compressional force in the outer shell of the tumour. The pattern of necrosis in tumour RIB_5 is further confirmation of the explanation advanced here. Necrosis develops in many parts of the central zone of tumours once they have attained a diameter of 7 mm, but none is evident at the periphery. At a later stage in growth, massive central coagulative necrosis occurs and the scattered areas of necrosis are

limited to a transitional zone outside the necrotic centre and within the well vascularised outer shell (Thomlinson [1] and personal communication). Thus unlike SSB1A, tumour RIB_5 seems to confirm with John's hypothesis [1]: "massive necrosis in tumours is due to venous infarction following slowly progressive venous obstruction consequent upon the expansion of the tumour in a limited space."

(b) *The capillary network*

The suggestion that a capillary network exists uniting the visible afferent and efferent vessels, but closing at death, was made in the description of the vascular pattern of tumour SSB1B and it was further suggested that such a network exists in all four tumour types. Without some such system it is clearly impossible for blood from the afferent vessels to reach the efferent. That such an important structure should function in life and become almost invisible at death is not surprising in view of the behaviour of the blood vessels revealed in a sandwich preparation, e.g. [12]. There, erythrocytes may be seen to pass through a capillary one at a time, but suddenly the flow may increase, only later to cease as suddenly leaving no indication of the vessel's existence. Groups of corpuscles may remain motionless together and then resume movement in a capillary that has suddenly become patent. Eddy and Casarett [21] reported that in their sandwich preparations of a rat neurilemmoma preliminary data showed that a negative pressure on the cover of as little as three inches of water (5.6 mm Hg) could restore the flow in an occluded preparation and as much as 12 in. of water (22.5 mm Hg) positive pressure on the cover was needed to occlude an actively circulating tumour.

In SSB1B and the other types of tumour, the visible evidence in favour of a capillary network is the existence of a faint grey reticulum, the clarity of which varies in different parts of the tumour, but which in the most favourable areas seems to unite afferent and efferent vessel endings. Erythrocytes, often flattened, are irregularly present on it. The reticulum could alternatively be interpreted as a pattern of cell membranes, but since it is sometimes continuous across partial breaks in a section, this seems unlikely and it is more probable that with the drop of arterial pressure at death, the network has almost completely closed, the erythrocytes being either forced into the efferent vessels, or trapped or broken within the network. If broken, their contents would escape and when stained with benzidine and hydrogen peroxide, give a weak positive response.

In spite of the doubt about its existence, it is possible to visualize the network sufficiently well to estimate its density. In all four tumour lines, this was found to be about 3000 capillaries per sq mm in fixed preparations, equivalent allowing for shrinkage to 2400 in fresh. The pattern seemed always to be related to cell shape and so varied little in different areas of the same tumour.

The distance for which the capillary network appears to be the sole channel for the transport of oxygen varies considerably in the four tumour lines. In the lax tumours, in the size range of tumours investigated, the distance from the apparent endings of the afferent vessels to the apparent start of the efferent does not exceed 250 μm and is usually less. Marked hypoxia therefore would not be expected and this conclusion is in agreement with the finding that massive central necrosis does not occur even in tumours of 50 mm dia (Howlett, personal communication). The occurrence of scattered islands of necrosis in both types of lax tumour suggests, however, that not all cells are fully oxic, and the response to radiation described later, shows that RIB_5C in particular contains a significant hypoxic cell fraction. This may in some way be connected with its partially peripheral vascular supply.

In the two tense tumours, the situation is altogether different since in them the capillary network provides the sole channel of transport from the periphery of the tumour to its centre, a distance of 4 mm in the size range of tumour investigated. Hypoxia seems certain to occur and this conclusion agrees with the finding that massive central necrosis occurs in both.

(c) *Hypertrophy of the venous system*

A characteristic of all four tumour lines, already commented on, is the extreme preponderance of venous blood. This hypertrophy of the efferent system affects the number of vessels as well as their diameters and is considerably greater in the tense tumours than in the lax. As a result, a superficial examination often suggests that a tumour is an exceptionally well vascularized structure compared with the tissue around it. It is only when the arteries are identified with certainty, either from the thickness of their muscular investiture if such exists, or by tracing them back to their origin, that it becomes apparent that the supply of oxygenated blood to some parts of the tumour is relatively less than to the adjacent skin, muscle or connective tissue. It seems likely that the cause of the disparity between afferent and efferent vessels is

the exceptional mode of origin of the tumour vasculature. There seems to be general agreement that, apart from the host vessels incorporated into the tumour as it expands, tumour vasculature arises by venous budding of the host vessels, followed by branching and anastomosis with the consequent formation of a network, which becomes connected with the afferent system only at a later stage. The weight of evidence in favour of Folkman's tumour angiogenesis factor [22] in evoking this venous budding is considerable. If this explanation is accepted, the first step in inducing the venous hypertrophy is the action of the T.A.F. on the endothelial lining of the veins surrounding the tumour. The disparity is then increased further in the tense tumours by the forces already described, i.e. differential stretching in SSB1A leading to the development of the greatly expanded circumferential veins and venous constriction in RIB_5 leading to the development of the central sinusoids.

Two structural features which might possibly be interpreted as measures of T.A.F. effectiveness are: (a) the spiralling of veins adjacent to the tumour and (b) the distance from the tumour of the point where a vein is deflected towards it from its original course. The resin embedded preparations show that for tumours of equal volume, both effects are more marked with tense tumours than with lax. It may, therefore, be that a further basic difference between the two types is that the former produces more T.A.F. than the latter.

An important deduction to be made from the existence of the marked venous hypertrophy is that an estimate of the total blood volume of the tumour, e.g. in assessing the extent of neovascularisation following irradiation, is not an effective measure of the oxygen supply, since a large, but variable, proportion of the blood is stagnant. For reliable assessment of oxygen supply, the essential requirement is a knowledge of the size and pattern of the afferent vessels.

(d) *The relation between vascular pattern and radiation response*

If the vascular pattern of a tumour is such that some of its cells are inadequately supplied with oxygen, since the hypoxic fraction is more resistant to radiation than the remaining neoplastic cells, radiation will be less effective than in a tumour where the pattern has given rise to a uniformly oxic population. "The response of... tumours to irradiation appears to be a sensitive indicator of the proportion of the cell population that is protected from radiation injury because of hypoxia" [15]. Detailed descriptions of the methods for determining the hypoxic fraction from the results of radiation treatment have been given by Thomlinson [3, 4, 5, 15], McNally [6, 7] and Howlett *et al.* [8]. Figure 8 has been redrawn from [8] plotting to the same scale the response of the four tumour lines considered here. The graph shows the relative delay in tumour growth produced by varying doses of 250 kV X-rays, relative delay being defined as the time taken for treated tumours to grow from 9 to 25 mm dia expressed as a multiple of that taken by untreated tumours. From earlier work using rats irradiated (a) while breathing air, (b)

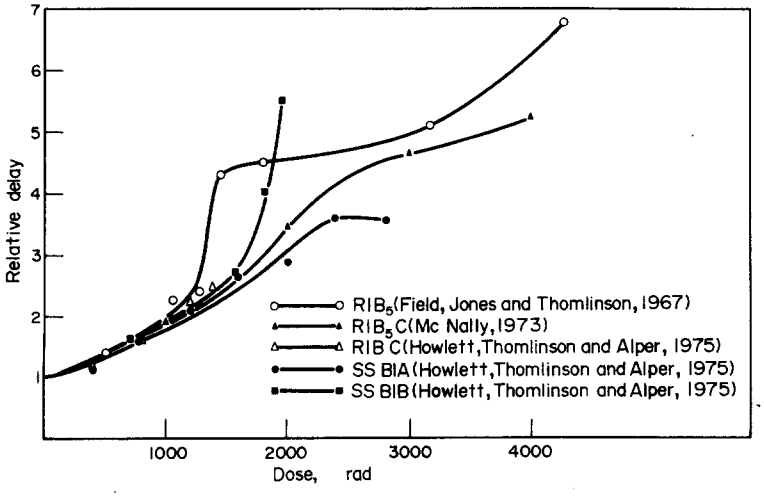


Fig. 8. The relative delay imposed on the four tumour lines by varying doses of 250 kV *X-rays, relative delay being defined as the time taken by treated tumours to grow from* 9 *to* 25 mm *dia expressed as a multiple of that taken by untreated tumours.*

while breathing oxygen at three times atmospheric pressure, and (c) while breathing normal air, but with the circulation to their tumours clamped so that all cells were effectively anoxic, and measuring either relative delay or survival of cells from tumours excised immediately after irradiation and cultured *in vitro*, it has been found that those tumours possessing a mixed cell population, both oxic and hypoxic, give a biphasic curve, when the results are plotted as in Fig. 8, while those with a uniform population give a monophasic curve. Of the four tumours considered here, the curves for RIB_5 and RIB_5C are clearly biphasic, while over the dose range employed, SSB1A appears biphasic and SSB1B monophasic, but in their cases high enough doses to justify a firm conclusion were not employed. In the case of SSB1B, however, Howlett *et al.* [8] quote additional earlier evidence and from it and from their own results conclude that no hypoxic fraction exists. For SSB1A no additional evidence of this kind is forthcoming, but recently Constable [10] has determined the pattern of oxygen removal rate in this particular tumour and reports that it was uniformly low throughout the core of the pre-irradiated tumour and more normal values were only found in a thin shell at the edge of the tumour. Though the oxygen removal rate of an excised portion of tumour outside the body of the host need have no exact relationship with the oxygen supply to the cells of the tumour *in situ*, this pattern agrees precisely with what has already been concluded from the study of the vascular pattern of the tumour.

To summarize, when the results of the available radiation experiments are compared with the expectations based on the patterns of vasculature, it is found that:

(a) there is complete agreement in the case of RIB_5 and SSB1B, the former having an hypoxic cell fraction, the latter not;

(b) RIB_5C, though having mainly internal supply and drainage, has an hypoxic cell fraction;

(c) the evidence from the radiation experiments is too incomplete for a firm conclusion to be drawn in the case of SSB1A, but if oxygen uptake rate is considered in its place, there is again agreement.

It can be concluded therefore, that in the four tumour lines considered, the pattern of vasculature is of greater significance than the relationship between tumour lines in determining response to radiation.

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Characterisation of Mammary Tumour Virus of Strain ICRC Mouse

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Abstract—*The mouse mammary tumour virus (MuMTV) isolated and purified from the milk of strain ICRC mice, susceptible to spontaneous development of mammary cancer and leukemia, was investigated for its morphological, biophysical, immunological and structural characters.*

The purified virions banded at the density of 1.17 g/ml *in* 0–55% *linear sucrose gradient and contain mature B type particles along with few C type particles when studied under the electron microscope. The polyacrylamide gel electrophoresis of strain ICRC MuMTV reproducibly resolved at least ten polypeptides. The mol. wt of the three major polypeptides are* 49,000 (*p* 49), 34,000 (*p* 34) *and* 24,000 (*p* 24). *Two of these polypeptides p* 49 *and p* 34 *have been characterised as glycoproteins on the basis of staining with periodic acid Schiff's reagent. High titers of MuMTV antigens were noted in the milk plasma of strain ICRC mice. The anti MTV-S serum could detect common antigens in disrupted virions of strains ICRC and C3H(Jax) by immunodiffusion test.*

Strain ICRC was a high cancer strain in the earlier generations of inbreeding (the average tumour incidence in the breeders of first ten generations was 81.3%) *however, the tumour incidence in the* 45th *generation has gone down considerably. The parameters studied in the present investigations have indicated that the MuMTV of strain ICRC exhibit characters comparable to those of high cancer strain C3H(Jax), in spite of a significant decrease in the mammary tumour incidence.*

INTRODUCTION

STRAIN ICRC developed at the Biology Division of Cancer Research Institute, Bombay, is susceptible to the spontaneous development of mammary cancer and leukemia, both the lesions having viral etiology. Twenty-five per cent of animals invariably develop both the lesions, breast cancer and leukemia together. Extensive investigations carried out on this strain for biological characteristics including hormonal factors and spontaneous tumour incidence have been published in series of papers [1–7]. The thin section electron microscopy of mammary tumours of females from early generations of inbreeding has shown presence of potent MuMTV in strain ICRC [8]. This strain was a high cancer strain during the initial generations of inbreeding. The average tumour incidence of mammary cancer and leukemia in the breeders of first 10 generations was as high as 83.9 and 38.2% respectively [3]. However, with continuous inbreeding, consistent steady decrease in the incidence of both the lesions was noticed, although the electron micrographs evinced the same amount of mature B particles [9]. At present the strain is in its 47th generation of brother sister matings and the tumour incidence for mammary cancer and leukemia in breeders of generations 45th are reported to be 20 and 16% respectively. On force breeding, however, the breast tumour incidence positively increases to 70%. Besides spontaneous leukemia, development of leukemias have been reported on innoculation of cell suspensions as well as cell free filtrates of mammary tumours of strain ICRC [10]. Such unique features about the strain and its changing tumour incidence warranted further studies on characterisation of its viral agents—MuMTV and MULV. The present communication reports studies on morphological, immunological and structural characters of MuMTV of strain ICRC. Since sufficient quantities of B particles can be obtained from the milk, it was of interest to carry out these

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studies on MuMTV isolated and purified from the milk.

MATERIAL AND METHODS

Isolation of virus

Milk from strain ICRC breeders (7th and 12th day after parturition) was obtained by vaccum suction. The virus was isolated and purified from the milk according to the method described by Sarkar and Dion [11]. The method is as follows:

To a pool of 4 ml ICRC mouse milk, 16 ml 0.15 M EDTA, pH 7.4 and 10 ml phosphate buffered saline (PBS), pH 7.4 were added and thoroughly mixed. The mixture was centrifuged for 10 min at 5000 rev/min. After discarding the cream fraction and the pellet, the skimmed milk was centrifuged for 1 hr at 21,000 rev/min in SW 25.1 rotor. The supernatant was slowly removed and the pellet was allowed to soak in 0.5 ml PBS overnight at 4°C. The resuspended pellet was then layered on to a preformed linear sucrose gradient (0–55%) in a 14 ml tube and centrifuged for 1 hr at 113,000 ***g*** in SW 40 rotor. In most cases two distinct bands were visible in the gradient tubes. The bands were either collected separately or together by the downward displacement, diluted with PBS and pelleted for 30 min at 160,000 ***g***. The resuspended pellets were applied to a 2nd preformed sucrose gradient as mentioned above. The visible bands were collected, diluted with PBS, pelleted, suspended in PBS and were then used for further studies.

The MuMTV, isolated and purified in the similar manner, from strain C3H(Jax) milk (MTV-S) was used for the comparative studies.

Negative staining

A drop of either milk or purified virus preparation was applied to a carbon film on a 200 mesh grid and allowed to sit for 15 min for the virions to adhere. The excess fluid was drawn off with filter paper and a drop of 2% phosphotungstic acid (PTA, pH 7.4) was applied to the grid for 30 sec and removed. Fixation of the virus was performed prior to staining by floating the grid with absorbed virus on a drop of 2.5% glutaraldehyde for 3–5 min. After 3–4 washings in buffer, the grids were stained as above.

Thin sectioning

The viral pellets were fixed in 6.25% glutaraldehyde for 1–1½ hr, post fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in ethanol solutions and embedded in araldite. The sections were cut on a Porter-5-Blum MT2 sorval ultramicrotome, stained in uranyl acetate followed by lead citrate and examined in a Philips 300 electron microscope.

Antisera

Standard anti-MTV-S serum (rabbit antiserum against DBA/2 MTV) was kindly provided by Dr. H. Tanaka, Virus Research Institute, Kyoto, Japan. The antiserum was absorbed with 2 vol of whole milk from C57BL mice for 1 vol of serum by incubation at 37°C for 30 min, storage at 4°C overnight and finally clarification at 200,000 ***g*** for 30 min [12]. The serum was also absorbed with 50 mg/ml of lyophilized tissue extract from C57BL mice incubation at 37°C for 1 hr and finally clarification at 200,000 ***g*** for 30 min.

Immunodiffusion tests

Double diffusion tests [13] using 0.7% agar in normal physiological saline containing 0.001% thiomersal was employed to test the ICRC MuMTV. Slides were incubated at room temperature in a humidified chamber for 72 hr. After the development of precipitin lines, the slides were washed in PBS or normal saline and were photographed.

Immunoelectrophoresis

Immunoelectrophoresis was performed in 1% Noble agar in barbitol buffer, pH 8.6. After the addition of antigens, the electrophoresis was performed at 5 mA/slide for 90 min. After adding the antiserum, slides were kept in a humidified chamber till lines developed and treated as above.

Hemagglutination-inhibition assay (HAI) for viral antigens

HAI test of Sibal *et al.* [14] with slight modifications was employed for the detection of MTV antigens in the milk plasma of strains ICRC and C3H(Jax). Rabbit anti-MTV serum prepared by immunization with purified B particles from ICRC milk according to the method of Daams *et al.* [15] was used. The procedure for the absorption of the antiserum was the same as for standard anti-MTV-S serum, already mentioned. Sensitization of the sheep erythrocytes (SRBC) was done by coating purified virus (2 mg/ml) isolated from strain ICRC milk on to 2.5% suspension of tannic acid coated SRBC in PBS, pH 6.4, at room temperature for 10 min [16]. The milk plasma of low cancer strains ICRC/fC57, C3H/Mect and L(P) were used as controls.

Chemical analysis

The relative proportion of protein and RNA was ascertained by the measurement of O.D. at 280 and 260 nm [17]. The protein content was also measured by the method of Oyama and Eagle [18]—a modification of Lowry's method [19]. The RNA was estimated by the method of De Deken–Grenson and De Deken [20].

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed by method of Shapiro *et al.* [21]. Proteins were precipitated in 10% trichloroacetic acid (TCA) at room temperature for 15 min and then centrifuged at low speed for 15 min. The pellets were thoroughly drained, washed with 5.0% TCA and resuspended in 100 μl of 0.01 M sodium phosphate buffer (pH 7.8) containing 1% SDS and 1% β-mercaptoethanol and incubated at 37°C for 2 hr. After complete denaturation of the proteins the samples were mixed with 100 μl of 50% sucrose and 5–10 μl tracking dye (pyronin 0.25%), mixed thoroughly and applied on the gels. The gels contained 7.5% acrylamide, 0.25% bis acrylamide, 0.1% SDS and 0.1 M sodium phosphate (pH 7.8). Electrophoresis was performed at 3 mA/gel for 30 min followed by 3–3½ hr at 7 mA/gel. The gels were stained for 3–4 hr in 0.25% Coomassie brilliant blue in 40% methanol and 10% glacial acetic acid and destained by diffusion in 7% acetic acid and 5% methanol. The stained gels were scanned at 580 nm on a Densicord–Photovolt corporation, NY.

Glycoproteins were detected by periodic acid Schiff's staining [22]. Molecular weights of the viral polypeptides were estimated from their relative migration in gels compared to standard proteins electrophoresed in parallel gels. The molecular weight standards used were bovine serum albumin, mol. wt 68,000; ovalbumin, mol. wt 43,000, pepsin, mol. wt 35,000; trypsin, mol. wt 23,000; haemoglobin (Bovine) mol. wt, 15,000; lysozyme, mol. wt 14,400 and cytochrome C, mol. wt 11,700.

RESULTS

Buoyant density

Two light scattering bands were consistently observed when the ICRC milk pellet was applied to the 0–55% linear source gradient and spun at 113,000 ***g*** for 1 hr. The two bands were usually 3–5 mm apart, the lower band being broader than the upper band (Fig. 1). The upper and the lower bands when collected separately and layered on two separate gradients were found to band at the densities of 1.15 g/ml and 1.17 g/ml respectively. The milk from strain C3H(Jax) breeders, when run simultaneously with ICRC milk, consistently showed only one light scattering band at the density of 1.17 g/ml. When the spinning time was extended to 3 hr one single light scattering band was noticed in case of ICRC MuMTV (Fig. 2).

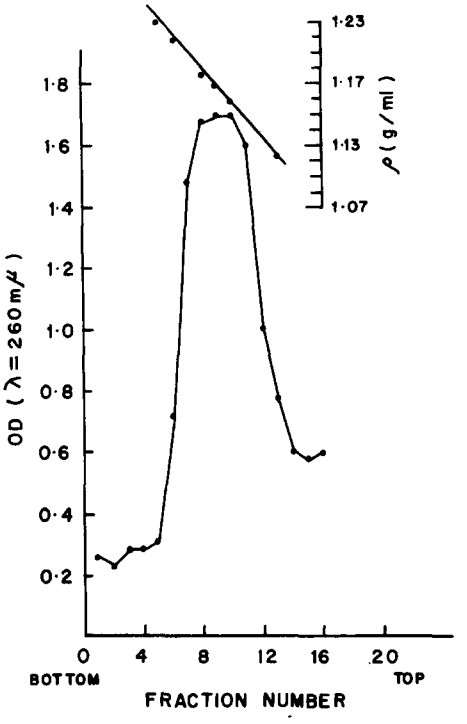

Fig. 2. Density gradient centrifugation of MuMTV from strain ICRC. The centrifugation was carried out for 3 hr *at* 30,000 rev/min *in Spinco SW* 40 *over* 14 ml *preformed sucrose gradient* (0–55%).

Virus morphology

In the negatively stained preparations of MuMTV, the virions showed striking morphological feature of the head and tail configuration (Fig. 3). When the virions were fixed with glutaraldehyde prior to PTA straining, practically all the particles became spherical and many of them were penetrated by stain revealing the core components of the virion. The characteristic surface projections were observed on most of the virions (Fig. 4).

In the thin sections, the purified viral pellet showed numerous mature B particles with occasional mature C type particles (Fig. 5). C type particles were noticed in the thin sections of lower as well as upper viral bands.

Immunodiffusion and immunoelectrophoresis tests

Standard anti-MTV S (DBA) serum was employed to test intact and disrupted B particles isolated from ICRC and C3H(Jax) milk. The serum produced one single line very near to antigen well with intact B particles from both the strains (Fig. 6). With ether disrupted B particles of both the strains, three precipitating lines appeared half way between the wells. All the antigens showed lines of identity in the immunodiffusion tests and β electrophoretic characteristics of B particle antigens in immunoelectrophoretic test.

HAI for viral antigens

It is evident from Table 1 that strains ICRC and C3H(Jax) had high antigen titers in their milk whereas the low cancer strains ICRC/fC57, C3H(Mect) and L(P) showed very low levels of MTV antigens.

Table 1. *Presence of MuMTV antigens in the milk of various strains of mice*

Mouse strain	Tumour incidence	HAI titer (Reciprocal)
ICRC	20%	1:256
C3H(Jax)	90%	1:1024
ICRC/f C57*	Nil	1:8
C3H/Mect†	Nil	1:32
L(P)	Nil	<1:8

*ICRC foster nursed on C57BL.
†C3H subline derived from C3H mammectomised females [47].

Chemical composition

On an average purified viral pellet obtained from 4 ml of strain ICRC milk was found to contain 3500 μg of proteins and 115 μg of RNA. Thus the total RNA content of virions was found to be 3.3% of the total proteins.

Polypeptide composition

The purified MuMTV from ICRC and C3H strains of mice was dissociated and electrophoresed as described before. Examination of the electrophoretic pattern after staining with coomassie blue revealed at least 12–14 polypeptide bands, 10 of which were detected reproducibly. The molecular weights of these as estimated from their relative migrations compared to standard proteins electrophoresed simultaneously were 100,000; 90,000; 81,000; 72,000; 62,000; 49,000; 34,000; 24,000; 15,000 and 12,000 (Table 2). Judging from the intensity of the stain polypeptides 49,000; 34,000 and 24,000 were considered as the major structural proteins of the virion (Fig. 7). The gels were

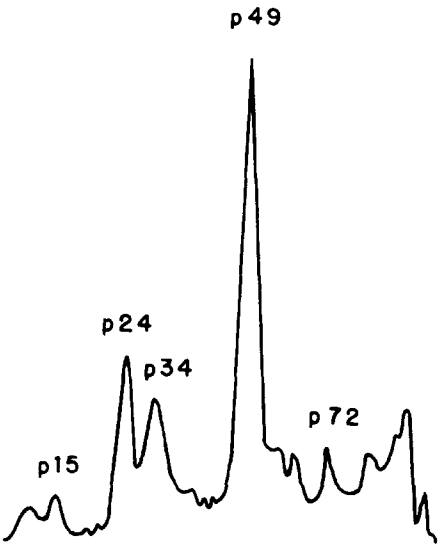

Fig. 8. *SDS-PAGE electrophoretic pattern of purified MuMTV from strain ICRC and the densitometric tracing of the gel stained with coomassie blue.*

scanned in densitometer and the relative percentage of each protein band estimated. Six gels were scanned for this purpose and average values were taken as representative of the relative amounts of individual polypeptides (Fig. 8). The same polypeptides were major or minor in each run but precise proportion varied among runs and strains. The three major polypeptides of mol. wt 49,000; 34,000 and 24,000 contained about 62.0% of the total protein. It should be mentioned here that we observed three more bands in the coomassie blue stained gels corresponding to the mol. wt greater than 100,000.

Six dye bands could be detected after staining of virus containing gels for glycoproteins with PAS (Fig. 9). These corresponded to polypeptides of mol. wt 100,000; 90,000; 62,000; 49,000 and 34,000. One additional band of glycoprotein was detected which corresponded to the polypeptide of the mol. wt more than 100,000. Thus it appears that the two major polypeptides of mol. wt 49,000 and 34,000 of MuMTV contain polysaccharides.

The relative proportions of carbohydrate in polypeptides of mol. wt 49,000 and 34,000 was observed to be more or less similar though the protein content of polypeptide of mol. wt 49,000 was found to be 1.8 times more than that of polypeptide of mol. wt 34,000.

DISCUSSION

MuMTV purified from milk or from mammary tumours have a characteristic buoyant

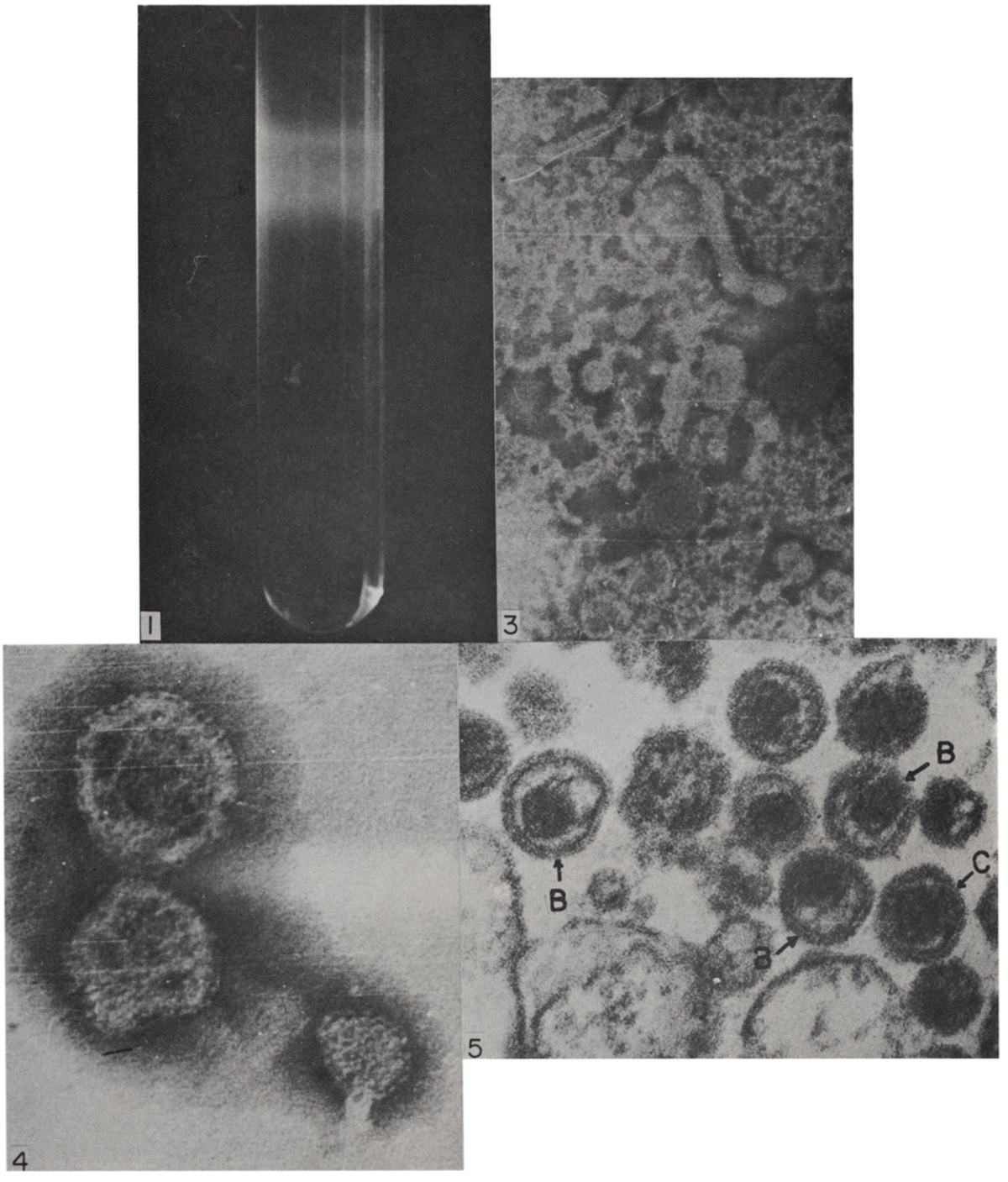

Fig. 1. *Sucrose gradient* (0–55%) *showing the location of MuMTV bands of strain ICRC. The gradient was overlaid with partially purified virus from* 4 ml *strain ICRC milk and was centrifuged for* 1 hr *at* 30,000 rev/min *in Spinco SW* 40 *rotor.*

Fig. 3. *Electron micrograph of strain ICRC MuMTV showing head and tail of the virion. Negative staining PTA prefixed.* ×313,500.

Fig. 4. *Electron micrograph of strain ICRC MuMTV. Negative staining PTA prefixed with gluteraldehyde.* ×380,000.

Fig. 5. *Electron micrograph of strain ICRC MuMTV showing mature B and C particles. Uranyl acetate staining.* × 156,750.

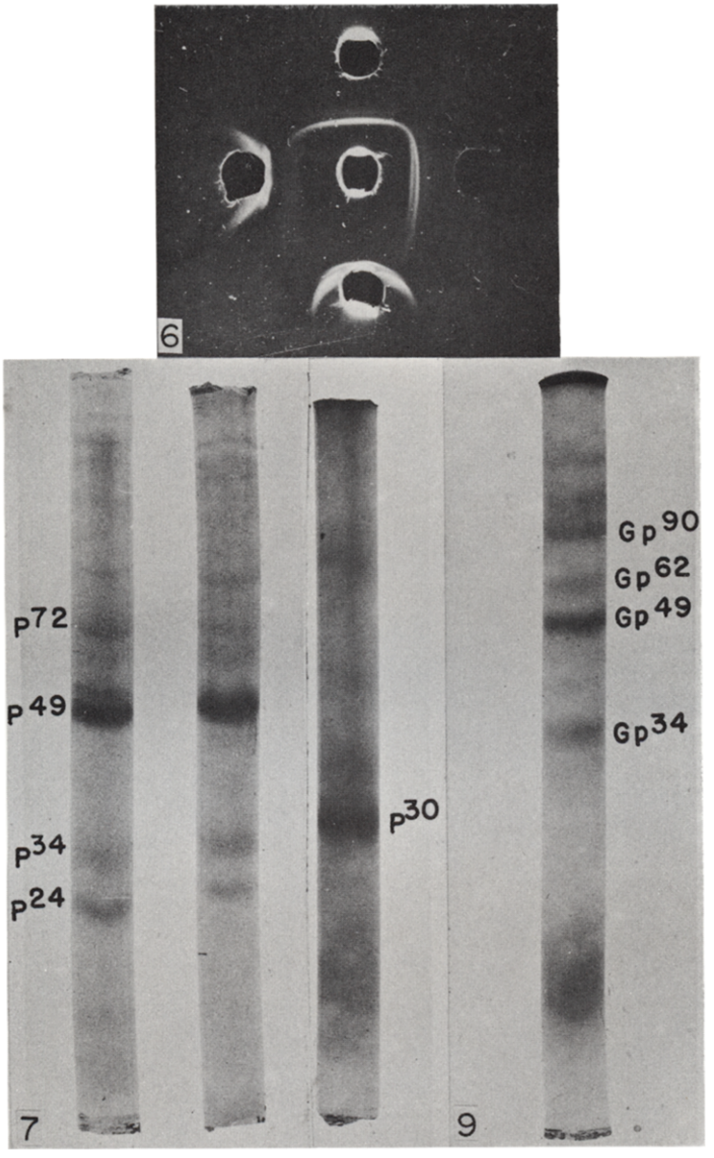

Fig. 6. *Immunodiffusion plate of strains ICRC and C3H (Jax) MuMTV tested against MTV-S aerum (Center well).*
Peripheral walls: Left—*ICRC B particles complete*, Top—*ICRC B particles ether disrupted*, Right—*C3H(Jax) B particles ether disrupted*, Bottom—*C3H(Jax) B particles complete.*

Fig. 7. *SDS-PAGE pattern of purified MuMTV of strains ICRC and C3H(Jax) and MuLV from strain ICRC stained with coomassie brilliant blue.*

Fig. 9. *SDS-PAGE pattern of purified MuMTV from strain ICRC stained with periodic acid–Schiff's reagent.*

*Table 2. The Polypeptides of MuMTV from ICRC mouse strain**

Polypeptide	Mol. wt ($\times 10^{-3}$)	Range	Relative percentage of the protein fractions
1	12	11–13	2.5
2	15	14–16	3.8
3	24	22–27	12.4
4	34 (GP)	30–35	17.0
5	49 (GP)	49–50	31.5
6	62 (GP)	60–64	4.8
7	72	69–73	10.6
8	81	78–82	4.5
9	90 (GP)	88–94	8.7
10	100 (GP)	98–101	1.9

*Average of six determinations.
GP Glycoprotein.

density in aqueous solutions which varies with the composition of the gradient [23, 24]. In sucrose gradient, the B particles band at the density of 1.17 g/ml. In the present study, where Sarkar and Dion method [11] was employed, two light scattering bands were consistently observed, one at the density of 1.17 g/ml and the other at 1.15 g/ml. In a simultaneous run with sucrose gradient, B particles from strain C3H(Jax) milk showed a single light scattering band at the density of 1.17 g/ml. The electron microscope studies in strain ICRC revealed the presence of B particles in predominance and a few C particles in each of the viral bands examined independently. This observation rules out the possibility of the upper band at the 1.15 g/ml. density being solely comprised of C particles though the presence of these particles in strain ICRC is of interest.

In a recent report by Sarkar, Manthey and Sheffield [25], on the effect of different preparative procedures for electron microscopy on the size and shape of both MuMTV and MuLV virions, a possibility of small particles present in MuMTV preparation which is intact and can carry virus infectivity has been suggested. Moore *et al.* [26] on the basis of a number of physical measurements also suggested that tumourigenic activity could be carried by a particle that was smaller than the usual B particles. In the present study the separation of B particles into two independent bands might be due to a difference in size of the virions resulting in a difference in sedimentation velocity and in the time needed to reach their density equilibrium. That this could be so is further strengthened by the fact when the gradient is subjected to extended spinning of three hours, the two bands merge into a single light scattering band. Further studies on the size differences of B particles in relation to their infectivity are underway.

The presence of C type particles besides B particles, in the milk of certain high mammary cancer strains of mice [27, 28] and in the culture medium of mouse mammary tumours has been reported by many [29–32]. Dmochowski *et al.* [27] have done the thin section electron microscopy of high speed centrifugal pellets of milk from different strains of mice with high or low incidence of mammary cancer and high or low incidence of leukemia. The presence of large numbers of B particles with few C particles in the milk of strain ICRC corroborates the findings reported by Dmochowski *et al.* [27] in the milk of strains of mice susceptible to mammary cancer with a medium incidence of leukemia.

MuMTV isolated from milk of strain ICRC was found to contain at least 10 polypeptides by polyacrylamide gel electrophoresis, with mol. wt ranging from 12,000 to 100,000 (Table 2). Three of these proteins, mol. wt 49,000 (p 49), 34,000 (p 34) and 24,000 (p 24) can be considered as the major structural proteins of the virions judging from the intensity of coomassie blue staining. Our identification of major polypeptides of MuMTV from ICRC milk agrees in general with those reported by others [11, 33–36]. However, certain differences are noted between their results and those obtained by us. The mol. wt of the major polypeptide according to our estimation and those reported by Dickson *et al.* [12] and Dickson and Skehel [37] is 49,000 whereas others have estimated values as 52,000 [35, 38, 39] and 55,000 [11, 40]. The same polypeptides are consistently major or minor though variations are observed in the precise proportions of different polypeptides reported by different workers in spite of a similar technique being used. These apparent discrepancies are

attributed to the differences in the sources of the virus [11]. In the present study, although certain minor differences are observed in the relative amounts of different mol. wt proteins of MuMTV from ICRC and C3H strains, both the strains contain p 49, p 34 and p 24 as the major polypeptides.

Polypeptide p 49 is the most prominent protein common to MuMTV isolated from strains R III, C3H, GR, A and BALB/c [11, 36, 37] and therefore considered to be a major group specific antigen (gs-1). It was shown to be a glycoprotein by PAS staining, glucosamine incorporation and lactoperoxidase iodination of intact virions [34, 35]. Further, Sarkar and Dion [11] on the basis of their immunodiffusion and immunoelectronmicroscopic studies convincingly established p 49 as a group specific antigen present on the virion surface and p 28 as the major group specific internal antigen (gs-2). In the present study we detected the presence of carbohydrate moieties in p 49 and p 34 by PAS staining in viral isolates of the both strains ICRC and C3H(Jax) suggestive of their locations on the virion membrane.

Since the MuMTV isolated from ICRC milk exhibited presence of few C type particles under the electron microscope, a comparison was made of the polypeptide pattern of MuMTV from strains ICRC and C3H(Jax) with a view to examining if some of the polypeptides attributed to ICRC MuMTV are derived from contaminating C type of viruses. In the polyacrylamide gels, the polypeptide pattern of MuLV (C type particles) isolated from strain ICRC leukemia lesions showed a major polypeptide of mol. wt 30,000 (Fig. 7) which was also reported by Oroszlan *et al.* [41] in MSV/MuLV viral preparations and was found to be a major group specific antigen. No protein corresponding to this major protein of MuLV was observed in the MuMTV of strain ICRC. This observation is further supported by the report of Kimball *et al.* [36], who observed the characteristic pattern of polypeptides of MuMTV only, in viral isolates from tissue culture fluid of primary cell cultures of BALB/c mice mammary tumours contaminated with MuLV to the extent of 3%.

The standard anti MTV-S serum could detect common antigens in the MuMTV isolated from ICRC and C3H(Jax) milk, in double immunodiffusion. The intact virions of both the strains produced a single strongly curved line very close to the antigen well indicating the particles to be of very large size [15, 42]. Blair [43] and Daams *et al.* [15] have reported complete B particles in this line when seen under the electron microscope. The anti MTV-S serum could produce 3–4 precipitating lines with ether disrupted B particles from ICRC as well as C3H(Jax) strains. All these lines show lines of identity. In immunoelectrophoresis experiments, these lines have β electrophoretic characteristics. Daams *et al.* [15] have done elaborate studies on antigenic structure of murine mammary tumour viruses in double immunodiffusion. These authors were able to show about three precipitating lines with disrupted MTV-S in LKB ouchterlony plates when tested against anti MTV-S serum. The effect of trypsin, pepsin, HCl, RNase, phospholipase C and acridine orange on the appearance of precipitation lines of MTV-S antigens was investigated. On the basis of these results, it was concluded by these authors that the lines towards the antiserum well represent the antigens of the membrane whereas the lines towards the antigen well represent the antigens of the nucleoid [44]. In the present studies, since the lines representing nucleoid and the membrane antigens show lines of identity in strains C3H(Jax) and ICRC, they indicate group specific antigens. Further work on the purification of subviral components of strain ICRC MuMTV for the antigenic studies is in progress.

In the present studies, we have employed HAI test of Sibal *et al.* [14] for the detection of MuMTV antigens in the milk plasma of strains ICRC and C3H(Jax). Using this method, these authors [14] have shown correlation between the mammary tumour incidence, presence of B type particles and the hemagglutination inhibition titer. Their findings were confirmed by Parks *et al.* [45]. By using more precise method like radioimmunoassay Verstraeten *et al.* [46] have studied the expression of MuMTV antigens in milk of female mice of several strains and substrains. It was concluded by these workers that the viral antigen levels were always more in the milk of high mammary cancer strains like C3H, GR and R III as compared to those of moderate mammary cancer strains like C3Hf, DBAf and low mammary cancer strains Af, DDf, 020 etc. In the present studies, both the strains C3H(Jax) and ICRC have shown comparable expression of viral antigens in their milk plasma. Although the mammary tumour incidence in breeders of strain ICRC has gone down to 20%, the parameters studied in the present investigations have indicated that the MuMTV of strain ICRC exhibit characters comparable to those of the high cancer strain C3H(Jax). Experiments are in progress to estimate the MTV antigens in the milk plasma by using

more precise methods to confirm these findings.

The strain ICRC is rather unique in having a large amount of MTV and still a low mammary tumour incidence. The decrease in the mammary tumour incidence in this strain can be therefore attributed to some other factors. The rise is the tumour incidence from 20 to 70% on force breeding is a point worth noting. Essentially it is the potency of hormonal stimulus that seems to determine the mammary tumour incidence.

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Effect of Methotrexate and Aminopterin on Tritiated Nucleoside Suicide of Colony Forming Cells, *in Vitro**

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Abstract—*The effects of methotrexate (MTX) and aminopterin (AMN) on the incorporation of deoxyuridine (dUrd), deoxythymidine (dThd) and uridine (Urd) into granulocytic/monocytic and lymphocytic colony forming cells (CFG-GM and CFC-L) from BDF_1 mice is demonstrated by the alteration of the tritiated nucleoside suicide of precursor cells. The proportion of colonies that survived suicide effects, i.e. self-irradiation, from a specific tritiated precursor reflects the number of CFC not incorporating the precursor. Preincubation of CFC-GM with MTX and AMN (10^{-5} M or 10^{-6} M) completely prevented* 6- ^{3}H-*dUrd* (5μCi) *suicide, partly reduced* ^{3}H-*dThd* (5 μCi) *suicide, and did not affect* ^{3}H-*Urd suicide when cells were pulsed for* 2–3 *hr. When cells were exposed to drugs and tritiated compounds for the duration of the culture period* (7 days), *the reduction in* 6- ^{3}H-*dUrd suicide by the folate antagonists* (10^{-7} *M*–10^{-8} *M*) *differed between CFC-GM and CFC-L. Results are discussed with respect to the suicide technique as a novel biochemical probe in the analysis of drug effects on colony forming cells*, in vitro.

INTRODUCTION

THERE has been much recent information on the mechanism of action of methotrexate (MTX), a folate inhibitor, *in vivo*, [1, 2] and *in vitro*, in many cell lines [1, 3–8]. Because this agent's hemopoietic toxicity is a major consideration in chemotherapy, we examined how MTX and aminopterin (AMN), an analog of MTX, alter the incorporation of various nucleosides into hemopoietic precursor cells. Our investigative approach combined colony forming cell (CFC) assays for the granulocytic/monocytic (CFC-GM) and B-lymphocytic (CFC-L) precursor cell lineages with the suicide technique, i.e. the lethal irradiation of a cell resulting from incorporation of a radioactive nucleic acid precursor. Our use of several tritiated precursors expands the scope of the suicide technique, which in the past was mainly associated with incorporation of radioactive deoxythymidine only [8].

Many [3–6] have directly determined radioactive nucleoside levels in cells after MTX treatment, but this direct analysis is not feasible in hemopoietic precursor cells for the following reasons: (1) physical isolation of a precursor cell population is not yet possible and (2) assuming that morphological identification could be established (for discussion, see [9]), the infrequency of these cells within the entire hemopoietic cell pool (about 2–5 precursor cells per 10^3 nucleated cells, according to our clonal assays [10]) adds to the impracticality of this direct approach. Hence, in our system, if MTX or AMN interferes with the incorporation of a specific radioactive nucleoside into a precursor cell, this effect would be reflected in the enhanced number of surviving colonies, i.e. progeny of precursor cells that did *not* undergo suicide. Thus, we studied how various dose regimens and drug incubation periods of MTX and AMN reduced the suicide effects of the following tritiated nucleosides: deoxythymidine, deoxyuridine and uridine, on CFC-GM and CFC-L development.

MATERIAL AND METHODS

BDF_1 female mice (Jackson Laboratory, Bar Harbor, Maine) were used as sources of bone

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marrow and spleen cells for the clonal cultures. Administration of methotrexate (MTX) (donated by Lederle Laboratories, Pearl River, New York) or aminopterin (AMN) (Sigma, St. Louis, Missouri) preceded doses of radioactive compounds (expressed as μCi per ml) by 1–2 hr in washed out drug studies or by 10 min in unwashed studies. Nonradioactive cytidine (Cyd) (Sigma) was also administered with MTX as a pretreatment. The following tritiated precursors were purchased from New England Nuclear (Boston, Massachusetts): methyl tritiated deoxythymidine (^{3}H-dThd) (sp. act. = 20 Ci/mM), 6-tritiated deoxyuridine (6-^{3}H-dUrd) (sp. act. = 30 Ci/mM), 5- and 6-tritiated uridine (5-^{3}H-Urd and 6-^{3}H-Urd) (sp. act. = 25 Ci/mM). 5-tritiated deoxyuridine (5-^{3}H-dUrd) (sp. act. = 16 ci/mM) and 5,6-tritiated uridine (5,6-^{3}H-Urd) (sp. act. = 16 Ci/mM) were both obtained from ICN (Irvine, California).

Unwashed drug studies

Bone marrow cells (1×10^5 cells) were plated in 0.5 ml McCoy's 5A media (modified) with 10% fetal calf serum (FCS) and 5% horse serum in 35 mm tissue culture dishes; spleen cells (1×10^5 cells) were plated in 0.5 ml minimum Eagle's medium with 20% FCS + 1% sheep red blood cells. Then drugs and radioactive agents were added to these cell suspensions. An agar-media admixture was then added to make 1.0 ml semi-solid media, such that the final concentration was 0.3% of Bactoagar. Bone marrow cells were cultured for CFC-GM according to our modification [10] of the technique of Metcalf and Moore [11]. Spleen cells were cultured for B-lymphocytic CFC, using the method of Metcalf *et al.* [12]. Cultures were examined for clonal growth after 7 days incubation at 37°C.

Washed out drug studies

Higher concentrations of the drugs and radioactive agents were used in washed out studies than in unwashed experiments, due to the duration of drug exposure, i.e. 4 hr and 7 days, respectively. Bone marrow and spleen cells were plated at 1 or 2×10^5 cells per well in 1.0 ml media + serum (as described for unwashed studies). Preliminary tests showed that the presence of serum during drug exposure did not significantly alter drug or suiciding effects, yet was optimal for maintaining cell viability (not shown). Cells were incubated with drugs and nonradioactive agents at 37°C, washed out and cultured in the same Costar 6-well cluster tissue culture dishes in order to eliminate cell suspension transfer steps and to reduce interference by density inhibition conditions during drug pulse. These culture dishes consisted of six 35 mm wells per cluster dish. After a designated drug preincubation period, cells were pulsed with radioactive nucleic acid precursors for 2–3 hr. A mixture of nonradioactive deoxynucleosides (10^{-5} M) was added to cultures to terminate the radioactive pulse; this chase consisted of equal parts of deoxyguanosine, deoxyadenosine, deoxythymidine and deoxycytidine (Sigma). After the chase cells were washed twice with Hanks' Balanced Salt Solution + 5% calf serum; cells were centrifuged at 300 $\times$ ***g*** in between washes, using a Cooke carrier to hold each Costar cluster dish in the centrifuge. After the final wash cells were resuspended in 1.0 ml semi-solid media and cultured as was described for unwashed cultures.

RESULTS

Table 1 shows the reduction by MTX (10^{-5} M or 10^{-6} M) and AMN (10^{-5} M) of the suicide effects of 5 μCi ^{3}H-dThd or 6-^{3}H-dUrd on CFC-GM formation in a washed out experiment. Bone marrow cells were preincubated with one of the folate inhibitors for 2 hr before exposure to a tritiated deoxynucleoside for an additional 2 hr; cells were then washed out and prepared for culture. Because MTX and AMN block the conversion of deoxythymidylate from deoxyuridylate through an inhibition of the folate reductase, we predicted that MTX and AMN would block the metabolism of exogenous 6-^{3}H-dUrd. On the other hand, since dThd can be incorporated via the salvage pathway, ^{3}H-dThd suicide would not be directly affected by the metabolic interference of MTX or AMN. Results generally supported these notions; they are expressed as CFC per dish, percent of control and as a suicide ratio (radioactive treatment/nonradioactive treatment). The suicide ratio indicated the efficacy of drug prevention of suicide effects. For example, AMN treatment reduced 6-^{3}H-dUrd suicide, giving CFC values that were 79% of control; when comparing this CFC value to its corresponding nonradioactive value (AMN treatment alone), a suicide ratio of 0.96 is obtained. If the CFC value observed when cells were pretreated with MTX or AMN before the radioactive pulse (Columns B or C, Table 1) was *not* significantly different from the CFC value derived from the corresponding nonradioactive counterpart (Column A, Table 1), this indicated a dramatic reduction in the tritium suicide resulting from drug interference with the incorporation of a radioactive precursor. In other words, we are measuring if there is any significant difference between the CFC growth obtained with or without pulse, relative to drug

effects. The highest degree of suicide prevention by a drug would, then, occur when there is no difference between drug-treated cultures and drug-pretreated pulsed cultures.

MTX (10^{-5} M) caused the greatest reduction in 6-^{3}H-dUrd suicide of CFC-GM, raising the suicide ratio from 0.38 to 0.99; however, all drug treatments considerably reduced 6-^{3}H-dUrd toxicity, such that CFC values (Column B, Table 1) were *not* significantly different from their non-radioactive counterparts (Column A, Table 1). Although there was a greater inhibition of ^{3}H-dThd suicide when cells were pretreated with MTX, rather than AMN, these drug-pretreated cultures (Column C, Table 1) *were* significantly different from corresponding cultures exposed to the drug only (Column A, Table 1). Hence, the folate antagonists were more effective in blocking the effects of 6-^{3}H-dUrd suicide, than the effects of ^{3}H-dThd suicide, in CFC-GM.

To determine if MTX could similarly inhibit the intracellular metabolism of exogenous uridine, bone marrow cells were exposed to MTX (10^{-5} M) for 1 hr before a 3 hr pulse with 5 μCi 5,6-^{3}H-Urd, 6-^{3}H-Urd or 5-^{3}H-Urd. Cyd (10^{-4} M) was administered with MTX to one set of cultures to determine its role as a metabolic intermediate in the Urd pathway in CFC-GM, i.e., how Cyd altered ^{3}H-Urd suicide. Cultures were washed out and the resulting CFC-GM formation 7 days later, shown in Table 2, revealed that ^{3}H-Urd suiciding was relatively the same (>90% inhibition), regardless of the position(s) at which the tritium label was located. MTX, by itself, did not affect this ^{3}H-Urd suicide, but when cells were pretreated with Cyd in addition to MTX, a marked prevention of ^{3}H-Urd suicide was observed in the number of surviving colonies.

Data shown in Table 3 indicates that the metabolic fate of dUrd *is* reflected by differences in suicide effects when the tritium label occurs at the 5- or 6-position; one would expect 6-^{3}H-dUrd

Table 1. *MTX and AMN reduction of suicide effects of* 6-^{3}H-*dUrd and* ^{3}H-*dThd on CFC-GM*

Pretreatment	A. No pulse CFC/dish	(%)	B. 5 μCi 6-^{3}H-dUrd CFC/dish	(%)	s.r.*	C. 5 μCi ^{3}H-dThd CFC/dish	(%)	s.r.*
Control	327 ± 15	100	125 ± 4†	38	0.38	104 ± 17†	31	0.31
MTX 10^{-5} M	298 ± 16	90	295 ± 20	90	0.99	164 ± 9†	50	0.56
MTX 10^{-6} M	295 ± 27	90	273 ± 35	83	0.93	202 ± 11†	61	0.68
AMN 10^{-5} M	269 ± 9	82	275 ± 3	79	0.96	113 ± 5†	34	0.42

2×10^5 bone marrow cells received either MTX or AMN for 2 hr before 2 hr incubation with 5 μCi 6-^{3}H-dUrd or 5 μCi ^{3}H-dThd. All drugs were washed out and cells were resuspended in semi-solid media for culture. Cultures were examined for colony formation 7 days later. Values represent ± S.E. of 4 cultures.

MTX = methotrexate; AMN = aminopterin; CFC-GM = granulocytic/monocytic colony forming cells; 6-^{3}H-dUrd = 6-tritiated deoxyuridine; ^{3}H-dThd = tritiated deoxythymidine.

*s.r. = suicide ratio $\left(\frac{\text{radioactive treatment}}{\text{nonradioactive treatment}}\right)$.

†Values (Columns B or C) are significantly different ($P < 0.05$) from corresponding nonradioactive values (Column A), according to Duncan's new multiple range test, preceded by analysis of variance.

Table 2. *Effect of MTX and Cyd on* ^{3}H-*Urd suicide of CFC-GM*

Pretreatment	No pulse CFC/dish	(%)	+5 μCi 5-^{3}H-Urd CFC/dish	(%)	+5 μCi 6-^{3}H-Urd CFC/dish	(%)	+5 μCi 5,6-^{3}H-Urd CFC/dish	(%)
Control	394	100	6	1	12	3	16	4
MTX 10^{-5} M	386	97	26	6	11	2	19	4
MTX 10^{-5} M plus Cyd 10^{-4} M			368	93	310	78	299	75
Cyd 10^{-4} M	320	81						

Legend as for Table 1 except bone marrow cells were exposed to MTX (10^{-5} M) and/or Cyd (10^{-4} M) for 1 hr, followed by a 3 hr pulse with 5 μCi ^{3}H-Urd labeled in the 5-, 6- or 5,6-position. Agents were washed out. Values represent the mean of 3 cultures.

Cyd = cytidine, ^{3}H-Urd = tritiated uridine.

suicide to be greater than 5-^{3}H-dUrd suicide, since the nonradioactive methyl group replaces tritium at the 5-position. A 2 hr pulse of bone marrow cells with 5 μCi 6-^{3}H-dUrd reduced CFC-GM formation by greater than two-thirds; an equivalent dose of 5-^{3}H-dUrd did not affect clonal growth (suicide ratio = 1.08). Even at four-fold higher doses, 5-^{3}H-dUrd reduced colony formation by only a third. For this reason, 6-^{3}H-dUrd was always used for our suicide studies. Borsa and Whitmore [4] previously demonstrated higher levels of radioactivity in DNA when ^{3}H-dUrd was labeled at the 6-position, compared to the 5-position.

Tables 4 and 5 represent unwashed experiments, i.e. studies in which drugs and radioactive compounds were present for the entire culture period. Table 4 shows results of 6-^{3}H-dUrd suicide (0.5 μCi) on CFC-GM and the inhibition of suicide effects by low doses of MTX (10^{-8} M) or AMN (10^{-7} M and 10^{-8} M). Although the toxicity of MTX (10^{-8} M) and AMN (10^{-7} M) was similar (77 and 70% of control, respectively), AMN caused a greater reduction in 6-^{3}H-dUrd suicide than did MTX, i.e. 1.07 and 0.31 suicide ratios, respectively. The prevention of suicide effects by 10^{-8} M AMN was also more effective than MTX, lowering 6-^{3}H-dUrd toxicity to a suicide ratio of 0.66.

CFC-L formation demonstrated a greater sensitivity to the effects of AMN and MTX than did CFC-GM cultures when drugs were not washed out (Table 5). For example, CFC-GM cultures treated with 10^{-8} M concentrations of MTX or AMN were 77 and 111% of control, respectively (Table 4), compared to CFC-values, i.e. 40 and 39% of control, respectively (Table 5). In addition, MTX and AMN were equally toxic to CFC-L formation.

MTX and AMN (10^{-7} M and 10^{-8} M) effectively reduced 6-^{3}H-dUrd suicide (0.5 μCi) in lymphocytic colonies, such that suicide ratios ranged from 0.71 to 1.10. Cultures exposed to AMN (10^{-7} M and 10^{-8} M) or MTX (10^{-7} M) before the radioactive pulse (Column B, Table 5) were not significantly different from cultures given the corresponding drug treatment without pulse (Column A, Table 5), indicating a high degree of suicide prevention. Although some reduction in 6-^{3}H-dUrd suicide occurred with 10^{-8} M MTX pretreatment (i.e. 0.03 suicide ratio altered to 0.71 suicide ratio), the suicide prevention was not as marked as with the aforementioned drug treatments; that is, pulsed (Column B, Table 5) vs "no pulse" (Column A, Table 5) CFC values were significantly different from each other, relative to the 10^{-8} M MTX treatment. AMN also did not significantly alter suicide effects when administered at a 10^{-9} M concentration (see Column B vs Column A, Table 5). Hence, although variations in the toxic effects of or in the inhibition of 6-^{3}H-dUrd suicide by MTX and AMN were observed between unwashed CFC-GM and CFC-L cultures, both assays for hemopoietic precursor cells demonstrated sensitivity to the block on 6-^{3}H-dUrd metabolism by MTX or AMN at low physiologic concentrations.

Table 3. 6-^{3}H-*dUrd or* 5-^{3}H-*dUrd suicide of CFC-GM*

Radioactive pulse	CFC/dish	s.r.
Control	286	
6-^{3}H-dUrd 5 μCi	78	0.28
5-^{3}H-dUrd 5 μCi	308	1.08
10 μCi	228	0.80
20 μCi	210	0.77

Legend as for Table 1 except bone marrow cells (1×10^5) were not preincubated with a drug before the 2 hr pulse. Agents were then washed out. Experimental values represent the mean of 2 cultures; mean control value derived from 4 cultures.

Table 4. *MTX and AMN reduction of* 6-^{3}H-*dUrd suicide of CFC-GM*

Pretreatment	No pulse CFC/dish	(%)	+0.5 μCi 6-^{3}H-dUrd CFC/dish	(%)	s.r.
Control	231	100	26	11	0.11
MTX 10^{-8} M	178	77	56	24	0.31
AMN 10^{-7} M	164	70	176	76	1.07
10^{-8} M	258	111	172	74	0.66

1×10^5 bone marrow cells received either MTX or AMN 10 min before 0.5 μCi 6-^{3}H-dUrd (not washed out). After semi-solid media was added, cells were cultured for 7 days, then examined for colony formation. Values represent the mean of 2 cultures.

Table 5. *MTX and AMN reduction of* 6-^{3}H-*dUrd suicide of CFC-L*

Pretreatment	A. No pulse CFC/dish	(%)	B. +0.5 μCi 6-^{3}H-dUrd CFC/dish	(%)	s.r.
Control	477 ± 20	100	17 ± 1*	3	0.03
MTX 10^{-7} M	172 ± 7	36	172 ± 2	36	1.00
10^{-8} M	195 ± 4	40	139 ± 5*	29	0.71
AMN 10^{-7} M	178 ± 2	37	196 ± 5	41	1.10
10^{-8} M	190 ± 4	39	178 ± 3	37	0.93
10^{-9} M	536 ± 7	112	43 ± 5*	9	0.08

Legend as for Table 4 except 1×10^5 spleen cells + 1% SRBC were cultured (not washed out). Values represent the mean ± S.E. of 3 cultures.
CFC-L = lymphocytic colony forming cells.
*Values (Column B) are significantly different ($P < 0.05$) from corresponding nonradioactive values (Column A) according to Duncan's new multiple range test, preceded by analysis of variance.

DISCUSSION

By combining suicide techniques with clonal assays, we can measure the ability of methotrexate or aminopterin to block the incorporation of specific nucleic acid precursors into hemopoietic precursor cells. The proportion of colonies that survive when either folate antagonist interferes with the process of tritiated nucleoside suicide indicates the proportion of precursor cells that are sensitive to the toxic effects of these agents, i.e. their inhibition of (nonradioactive) nucleoside incorporation into DNA or RNA. To our knowledge, this study represents the first attempt to determine the extent of MTX or AMN damage to hemopoietic precursor cell as it relates to a specific nucleoside's incorporation.

To examine how a short (4 hr) pulse with MTX and AMN altered dUrd and dThd incorporation into CFC-GM, bone marrow cells were exposed to different concentrations of the drugs before receiving 5 μCi 6-^{3}H-dUrd or ^{3}H-dThd. These doses (10^{-5} M and 10^{-6} M) were in the range of serum levels (10^{-8} M–10^{-4} M) found in patients on a high-dose MTX schedule [13] and thus, were clinically relevant. Others [1, 4, 7] have shown MTX inhibition of 6-^{3}H-dUrd incorporation into mature cell lines from normal and transformed tissues. Results in Table 1 similarly show that MTX and AMN block the conversion of dUrd to dThd in granulocytic/monocytic precursor cells, for both agents reduced 6-^{3}H-dUrd suicide. However, because dThd can bypass the MTX and AMN block by means of the salvage pathway, we did not expect any significant inhibition in ^{3}H-dThd suicide by the folate antagonists. Tew and Taylor [14] noted that bone marrow tissue of rats was capable of circumventing the MTX block *in vivo* by means of the salvage pathway. Although a 2 hr pretreatment with MTX (10^{-5} M and 10^{-6} M) resulted in a greater reduction of ^{3}H-dThd suicide than with AMN (10^{-5} M) pretreatment, these differences were not statistically significant. Hryniuk [6, 7] attributed a decrease in ^{3}H-dThd incorporation in L5178Y cells to early MTX obstruction of *de novo* purine synthesis; the early "purineless" death was reversed with hypoxanthine. The mechanism for MTX inhibition of dThd incorporation was suggested to be due to an indirect impairment of the reductase through a block of RNA synthesis [6]. We are presently investigating the role of purines in conjunction with dThd in alleviating MTX toxicity to CFC-GM and CFC-L, using the suicide technique.

Results of Table 2 show that the suicide of CFC-GM was the same, whether labeled at the 5-, 6- or 5,6-positions; MTX by itself did not alter these suicide effects, but MTX + Cyd did markedly reduce ^{3}H-Urd suicide. On the other hand, others [4, 6, 7] reported a decrease in 5-^{3}H-Urd incorporation into RNA of passaged cells after MTX treatment and suggested that the MTX-induced purineless state was a factor in this reduction (see above Discussion). Taken together, these results suggest the following possibilities: (1) ^{3}H-Urd is incorporated into RNA of granulocytic/monocytic progenitor cells and causes their suicide, but MTX does not affect this incorporation as seen in other cell types [4, 6, 7]; or (2) ^{3}H-Urd suicide is due to its incorporation into DNA, which is not blocked by MTX. Some preliminary findings (unpublished) that cytidine, but not deoxycytidine, reduces ^{3}H-Urd suicide support the first interpretation; however, further studies are required. The observation that 5-^{3}H-Urd suicide was blocked when Cyd (10^{-4} M) was added with the MTX pretreatment (Table 2) suggests that the observed suicide reduction was most likely due to the effects of Cyd alone.

The greater effect of ^{3}H-dUrd suicide of CFC-GM that occurred when the isotope was labeled at the 6-position, rather than the 5-position, points out the sensitivity of the suicide technique to detect the metabolic substitution of tritium with a nonradioactive methyl group at the 5-position of the dUrd compound. The fact that 5-^{3}H-dUrd suicide *did* occur at all could be explained by the radioactive (as opposed to the chemical) impurity of the isotope, i.e. 25% of tritium label occurred at the 6-position (confirmed by ICN).

CFC-L formation was generally more sensitive to MTX and especially, to AMN toxicity than was CFC-GM when drug exposure lasted for 7 days (Tables 4 and 5); e.g. with 10^{-8} M MTX, CFC-GM and CFC-L values were 77 and 40% of controls, respectively. Although Pinedo *et al.* [5] reported an enhanced toxicity of MTX (10^{-8} M) to CFC, i.e. 50% inhibition, when dialyzed FCS was used (vs undialyzed), it should be pointed out that our assay employing undialyzed FCS was more sensitive to MTX toxicity than their assay using undialyzed FCS. Moreover, in our assay system, clonal cultures of P815 mastocytoma cells were completely inhibited by 10^{-8} M MTX (unpublished). It has also been shown that serum levels of $2–5 \times 10^{-8}$ M MTX were not clinically toxic 72 hr after MTX administration [13]. It is of interest that at 10^{-8} M concentrations, MTX alone slightly reduced CFC-GM values (77% of control), yet did not markedly affect 6-^{3}H-dUrd suicide (as was also observed in L5178Y cells [7]); whereas, AMN was not toxic to CFC-GM (111% of control) and decreased 6-^{3}H-dUrd incorpo-

ration, raising the suicide ratio from 0.11 to 0.66. Hence, the clinical implications are that, at 10^{-8} M concentrations (over 7 days), AMN was more effective in blocking methylation of dUrd in bone marrow precursor cells than MTX. However, 10^{-8} M MTX did reduce 6-^{3}H-dUrd suicide in CFC-L; others [1, 5] have observed a similar reduction in 6-^{3}H-dUrd incorporation into various cell lines after the same MTX dose. Thus, MTX and AMN prevention of 6-^{3}H-dUrd suicide of CFC-GM was similar when cells were exposed to either drug for a short period (Table 1), but when drugs were not washed out (Table 4), 6-^{3}H-dUrd suicide was decreased to greater extent by AMN than MTX.

The 10^{-7} M and 10^{-8} M concentrations of MTX and AMN decreased 6-^{3}H-dUrd suicide in CFC-L (no wash study), such that the suicide ratios ranged from 0.71 to 1.10 (Table 5). Only the 10^{-9} M dose of AMN failed to alter 6-^{3}H-dUrd suicide (suicide ratio of 0.08). AMN (10^{-8} M) appeared to be more effective in preventing 6-^{3}H-dUrd suicide than MTX (10^{-8} M), although this difference was less marked than was seen in CFC-GM cultures; this may be related to the fact that MTX and AMN were equally toxic to CFC-L.

In conclusion, we have shown how the suicide technique can be used to determine the biochemical status of hemopoietic precursor cells, especially as it relates to drug mechanisms. Unique advantages of the suicide technique as a biochemical probe are: (1) It is possible to employ tracer concentrations of nucleosides (because they are tritiated*) to detect the biological response, viz. suicide, to drug alteration of nucleoside incorporation into major cellular macromolecules involved with replication mechanisms (DNA). (2) The short drug pulses (terminated by washing procedures), by temporal definition, specifically affect the precursor cell population when measured by clonal assays. (3) Although the number of CFC's that were irradiated from ^{3}H-nucleoside suicide is indirectly determined (relative to controls), the suicide technique is the only practical means at present of measuring such biochemical properties of hemopoietic (or even tumor) precursor cells. Finally, the clinical applications of the suicide technique could be done with little, or no revisions of the existing technique. We have already conducted preliminary studies on tritiated nucleoside suicide in human lymphoblastic CFC (in preparation). Also, we have found that this technique has wide applications; similar concentrations of other folate reductase inhibitors, trimethoprim and pyrimethamine, as well as other anti-tumor agents, e.g. 6-mercaptopurine and 5-fluorodeoxyuridine, will prevent the suicide effects of appropriate tritiated nucleosides in clonal cultures (in preparation).

*2 μCi of ^{3}H-dThd (sp. act. = 20 Ci/mM) is approximately equivalent to 10^{-10} moles labeled compound.

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Kinetic Features of a Lymphoid Population in the Blastic Crisis of Chronic Granulocytic Leukaemia

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Abstract—*Kinetic and chromosome studies were performed on the primitive cells from a patient in the blastic phase of chronic granulocytic leukaemia who had two distinct cell populations. Lymphoblastic cells predominated and stained with fluorescent labelled anti-acute lymphoblastic leukaemic sera. There was variation in the proliferative activity of the myeloid population but the lymphoid cells remained essentially quiescent.*

A Ph^1 chromosome was identified in the myeloid but not found in the lymphoid cells.

The findings suggest the presence of a population unlike those of acute lymphoblastic leukaemia with limited metabolic capacity.

INTRODUCTION

THE PRESENCE of immature cells with characteristics similar to lymphoblasts has been described in patients during blastic crisis of chronic granulocytic leukaemia (CGL) [1–7]. Such cells can now be recognised not only morphologically but they have the membrane markers recognized by anti-ALL serum [8].

The kinetics of these cells have not been studied previously. In this paper we present some analyses of the proliferative behaviour of the leukemic cells in a patient with CGL in blastic crisis whose peripheral blood and bone marrow contained a mixture of primitive myeloid and lymphoid cells.

Clinical details

A 37-yr old man was found to have CGL while in hospital for the repair of an inguinal hernia. The white blood count was 385×10^9/l, platelets 414×10^9/l and haemoglobin 14.7 g/dl. The Philadelphia chromosome was seen in direct bone marrow examination. He was treated with busulphan intermittently. There was a marked improvement in the clinical and haematological condition and he remained well for 17 months. He then developed thrombocytosis in which the platelets were increased to over 1000×10^9/l, followed by an increased white blood count and the appearance of blast cells in the peripheral blood. Bone marrow and blood findings were consistent with a blastic crisis. There was a predominance (80%) of primitive cells morphologically resembling lymphoblasts which reacted with anti-ALL serum. As a result of the treatment which included vincristine and L-asparaginase there was a reduction in the total blast cell count together with an improvement in the patient's general condition. He then developed an infection which was accompanied by neutropenia. The bone marrow at this time showed persistence of both primitive lymphoid and myeloid cells. He remained relatively well for a period of five months. Eight months later blast cells reappeared in large numbers in the peripheral circulation and despite intensive chemotherapy he died 27 months after the original diagnosis.

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MATERIAL AND METHODS

Kinetic and chromosome studies on the bone marrow and peripheral blood specimens were performed at the beginning of and repeated throughout the blastic crisis.

Methods

Aliquots of bone marrow and peripheral blood samples were incubated separately at 37°C with 2.5 μCi/ml of 3H-TdR (specific activity 5 Ci/mmole) for half an hour, 5 μCi/ml of 3H-UR (specific activity 5 Ci/mmole) and 10 μCi/ml of ^{14}C-leucine (specific activity 348

mCi/mmole) for one hour and smears were made of each buffy layer.

The DNA content of the cells was measured by microdensitometry of Feulgen stained nuclei [9] and this was combined with autoradiography [10].

Chromosomes were studied on direct bone marrow specimens and in peripheral blood cells stimulated with and without phytohaemagglutinin according to the method of Moorhead *et al.* 1960 [11]. During the terminal crisis, primitive myeloid and lymphoid cells in the bone marrow were separated using a cell sorter by means of an anti-ALL serum [12, 13] and the nuclear DNA content of both populations of cells was examined by microdensitometry using a Vickers M85 Microdensitometer.

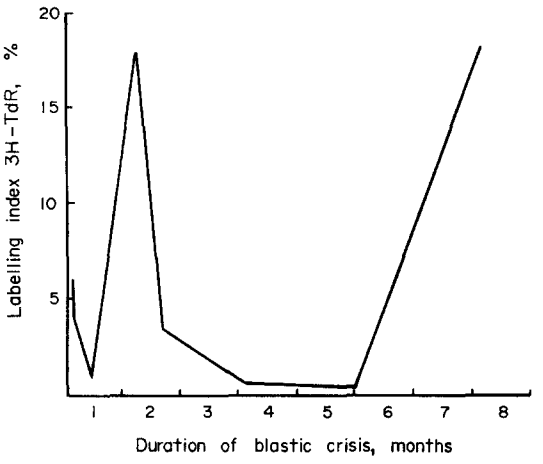

Fig. 2. Labelling index of large "myeloblasts" cells in the bone marrow during blastic crisis.

RESULTS

At the beginning of the blastic crisis, 80% of the nucleated cells in the peripheral blood and bone marrow were lymphoid (Fig. 1) and about 10% were myeblasts. There was a variation in the proportion of these cells throughout the course of crisis but lymphoid cells remained predominant. The majority of lymphoid cells were small (7–9 μ dia), with scanty cytoplasm and seldom had obvious nucleoli. They did not give a non-specific esterase reaction and some had periodic acid schiff (PAS) positive granules. The degree of PAS positivity did not change throughout the course of blastic phase. Some immature cells with a high cytoplasmic:nuclear ration were Sudan Black positive, along with the more clearly differentiated myeloid cells. It was not always easy to determine the origin of the medium sized blast cells. Although there were blocks of PAS positivity in some, others had the characteristics of myeloblasts. The peripheral blood LAP score was 17 (Normal control 15–95).

Labelling index (LI)

3H-*TdR*. At the beginning of malignant transformation the 3H-TdR uptake of the myeloblasts in the bone marrow was 4.5% increasing to 18% three weeks later. The number of cells in DNA synthesis fell to 4% in the next month and remained very low until the terminal stage when the LI of the myeloblasts rose to 18.4% again (Fig. 2). The LI of the lymphoid cells was $<1\%$ and remained low throughout the blastic crisis. Cells in mitosis were sparse (0.03%) and their number remained very low throughout the course of the blastic crisis.

3H-*UR*. The incorporation of 3H-UR was to measure the RNA synthetic activity. The myeloblasts had a high LI (80–86%) while only 5% of the small lymphoid cells had incorporated 3H-UR. The intensity of labelling examined by grain count was considerably higher in the myeloblasts (grain count 50–100) than in the small lymphoid cells (grain count 10–25). When size is taken into consideration the difference in grain count is unchanged.

^{14}C-*leucine*

At the onset of blastic crisis 88% of the myeloblasts incorporated ^{14}C-leucine while the LI of the small lymphoid cells was only 20%. A similar result was obtained at the terminal stage of the blastic crisis.

Lymphocyte transformation. No response to PHA occurred in the three day cultures of peripheral blood.

Chromosomes. The Philadelphia chromosome (Ph^1) was present in dividing myeloid cells on direct analysis of the bone marrow at diagnosis, in the beginning and throughout the blastic crisis. Lymphoblasts recognised by anti-ALL serum showed very few mitoses and we were unable to demonstrate the Ph^1 chromosome in any clearly recognisable lymphoid cells. Polyploidy and endoreduplication were seen in both direct bone marrow preparations and peripheral blood cultures during the blastic crises.

DNA content

Figure 3 shows the distribution of nuclear DNA content of lymphoid and myeloid cells in the bone marrow during the first month of blastic crisis. The lymphoid cells have a 2n DNA content with no cells in S while the myeloblasts

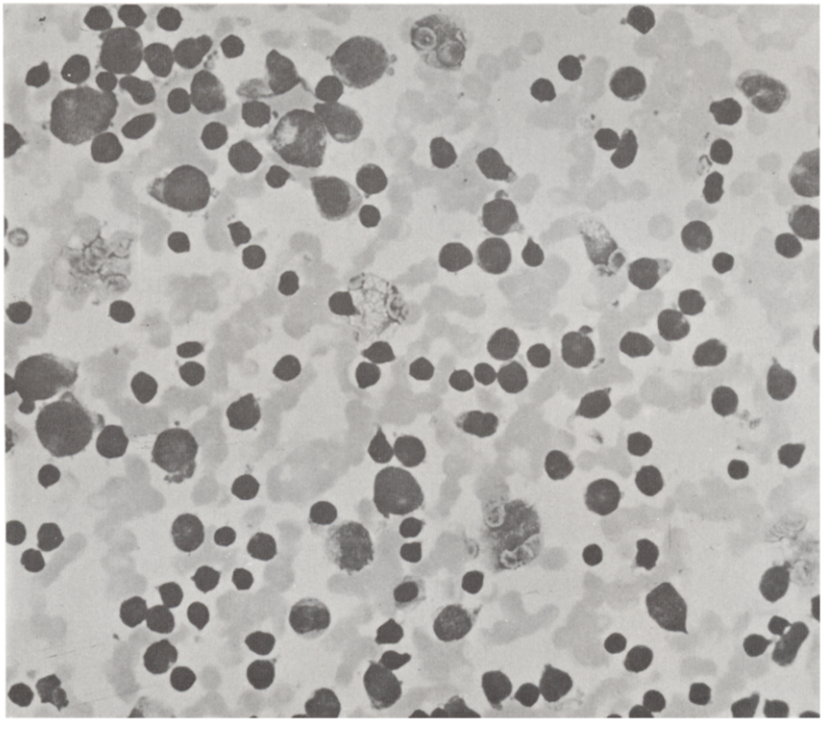

Fig. 1. Bone marrow stained with Jenner Giemsa demonstrating the presence of small "lymphoid" type cells.

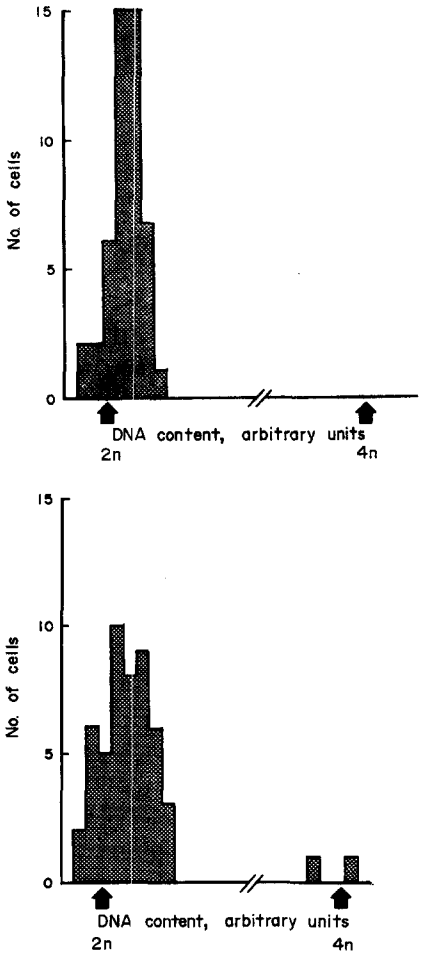

Fig. 3. Distribution of nuclear DNA content. Above small "lymphoid" cells and below large "myeloid" cells in the bone marrow.

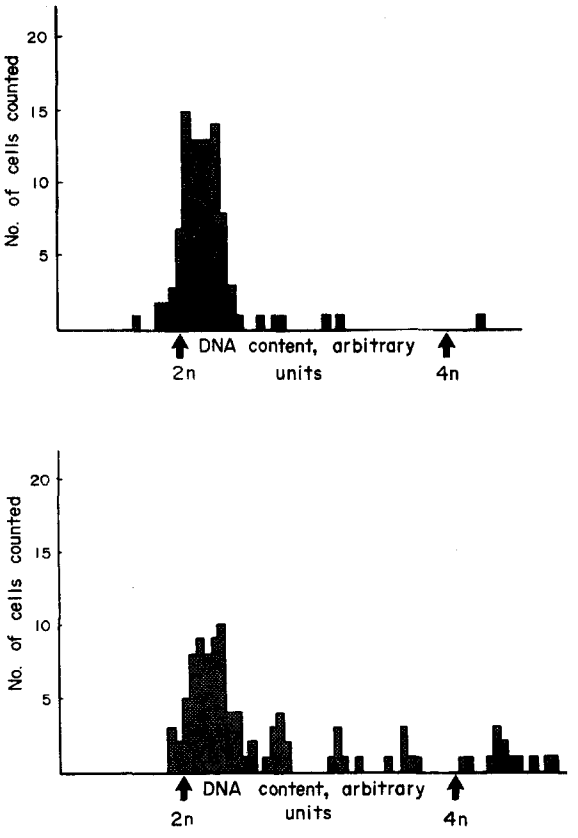

Fig. 4. DNA content of bone marrow cells separated by FACS (Fluorescence activated cell sorter). The upper histogram denoting ALL +ve and the lower ALL −ve cells.

have a wider distribution of unlabelled myeloblasts around G_1 with some in S phase.

When cells were separated on the cell sorter, according to their surface characteristics, there was no significant difference in the G_1 population between the ALL positive and ALL negative cells. However, in the myeloid cells not reacting with anti-ALL serum there were 18% of cells in S, G_2 and 8% with a DNA content above 4n (Fig. 4). The LI of myeloblasts at the same time was 18% while that of lymphoid cells was 1%.

Rosetting

The percentage of "T" "B" and "M" rosette forming lymphocytes was calculated in the peripheral blood. At the beginning of the blastic crisis, T and B cells were detected in reduced numbers and no M cells were present. A subsequent examination done one month later showed persistently low T and B rosettes.

DISCUSSION

It is well known that blasts from some cases of CGL in blastic crisis can morphologically resemble lymphoblasts [4, 6, 7]. The responsiveness of these cells to vincristine and prednisolone has been indirect evidence of their lymphoid origin. Recognition of such cells by anti-ALL serum [8] and the presence of the Ph^1 chromosome in some cases [7] supports this idea. It has been suggested that these blast cells arise from a pleuri-potential stem cell having the capacity not only to form erythrocytes, megakaryocytes and granulocytes but also the lymphoid population. This possibility was the subject of much argument among biologists around the turn of the century.

In the reports of cellular kinetics of bone marrow and peripheral blood from patients in blastic crisis of CGL, the emphasis has been on the study of the myeloid population, but the proliferative activity of lymphoid cells has not been investigated. Killmann *et al.* (1972) [14] found myeloblasts had a low 3H-TdR uptake during blastic transformation. A reduction in the number of cells synthesising DNA was observed in the bone marrow of CGL patients during blastic crisis [15, 16,

17]. The findings in our patient differ from others as there was a marked variation in the LI of myeloblasts during blastic crisis.

The blastic crisis of CGL has been compared with acute leukaemia [18], and the chronic phase of the disease considered as a preleukaemic state [14, 19, 20]. The kinetic behaviour of myeloblasts in our patient as demonstrated by 3H-TdR uptake and distribution of DNA content was shown to be similar to results obtained in acute leukaemia [15].

The fall in LI following chemotherapy and the rise terminally is similar to relapse of acute leukaemia [21] as are the variations in the distribution of nuclear DNA content [22] suggesting the presence of an aneuploid population of cells [23]. The latter was confirmed by chromosome studies.

The LI of the lymphoid population was low at the beginning of the crisis and remained so throughout the disease. The ability of the small lymphoid cells to synthesise RNA and protein was significantly less than the myeloid. The lymphoid cells had a 2n DNA content and none appeared in S or G_2. In repeated studies during the first two months the distribution of their DNA contents remained the same. In the terminal state the distribution of DNA content of anti-ALL +ve cells continued to differ from anti-ALL −ve (myeloid) cells. A few of the latter were in S or had a DNA content >4n. Theoretically the small lymphoid cells could either be in prolonged G_1 or out of cycle (G_0) with the potential to resume division once stimulated. The low RNA and protein synthetic activity is unlike the majority of blast cells in ALL in which the LI with 3H-UR is very high [24]. Certain points of dissimilarity between lymphoid cells in this patient and those of ALL are apparent:

(i) persistently low LI of blast cells with 3H-TdR throughout the course of the disease;
(ii) the lack of change in the distribution of DNA content usually seen in patients with ALL treated with chemotherapy;
(iii) very low RNA and protein synthetic activity.

We were unable to define a Ph^1 chromosome in the lymphoid cells on both direct bone marrow examination and peripheral blood, but the lack of sufficient chromosome spreads leaves this possibility unanswered. The kinetic pattern of the lymphoid cells containing Ph^1 has yet to be investigated.

The findings of different kinetic behaviour between the lymphoblasts in acute leukaemia and those presented here in blastic crisis indicate dissimilarity between the two populations. The lymphoid cells in this patient may be stem cells which fail to differentiate and accumulate in the bone marrow and peripheral blood [3]. It is suggested that in ALL the stem cells are amongst the circulating smaller lymphoblasts and the population is self-maintained [21]. The lymphoblasts described here appear to behave more like the stem cell in ALL and the explanation for their presence is unclear. Recent reports have shown that a small number of acute lymphoblastic leukaemias are in fact blastic crisis of chronic myeloid leukaemia [7]. If this is true then chemotherapy cannot be responsible for the emergence of this abnormal clone, although it may have been a contributory factor in this case. In view of the limited proliferative capacity of these primitive cells, it seems to us most likely that these cells have been present for some time and possibly from the beginning of the disease process.

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The Correlation of the Ongoing Proliferative Response to Tumour in Lymphoid Organs with the Anti-tumour Activity of the Lymphoid Cells

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Abstract—*Anti-tumour activity of washed lymph node (LN) and spleen cells of mice bearing a transplanted methylcholanthrene-induced tumour (MC1) for* 10–14 *or* 25–28 days *was compared and correlated with the stimulation of deoxyribonucleic acid (DNA) synthesis in the lymphoid organs. While the anti-tumour effect per lymph node cell (LNC) changed little if at all during this period, the stimulation of DNA synthesis in the total cell population of the nodes diminished significantly by the* 25–28 day *stage of tumour-bearing in contrast to the markedly high level seen at* 10–14 days. *In the spleen, however, stimulation of DNA synthesis in the total cell population increased throughout the period of study while the anti-tumour activity per cell significantly decreased. Depressed effector activity of spleen cells was shown to be associated with a decrease in anti-tumour activity of the T cell population. These observations provide evidence for a suppression of the immune response to tumour antigens operating at the level of sensitization of the immunocompetent cells and further suggest that proliferation in the tumour-bearing host of a cell clone(s) with suppressor function may have a direct or indirect role in controlling the specific response to tumours.*

INTRODUCTION

THE FUNCTIONAL roles of the various lymphoid cell types involved in the immune response to tumours and tissue grafts are complex. Thymus derived (T) cells, essential for the development of immunocompetence, are also involved in the regulation or suppression of the immune response. Thus it has been shown that spleen localising T cells can suppress the response in lymph nodes [1, 2]. *In vitro* studies have shown that splenic T cells suppress the mixed lymphocyte reaction (MLR) whereas lymph node T cells from the same donors enhanced the MLR [3]. In the experimental animal it has been repeatedly shown that splenectomy improves the immune response in favour of the tumour host [4, 5]. Furthermore, treatment of the host with tumour bearer spleen cells can enhance tumour growth [6–8]. These and other similar observations support the view that the immune response to tumours can become suppressed, although the exact mechanism of this is far from clear. It was observed previously [9] that stimulation of DNA synthesis in lymph node cells became markedly impaired during tumour bearing, suggesting suppression of sensitization and effector cell production in lymph nodes. It was further suggested that suppressor factor(s) liberated during the course of the immune response to the tumour could be responsible for this impairment. In the present investigation we have examined the progess of anti-tumour activity of the lymph node and spleen cell populations in relation to the stimulation of DNA synthesis in these organs to gain further insight into the nature of the suppressor mechanism.

Accepted 15 September 1977.

MATERIAL AND METHODS

The materials and methods have been described previously in detail [6, 9] and only variations or new techniques are described here.

Mice

Inbred BALB/c mice of MRC origin were obtained from Bantin & Kingman Limited, Hull.

Tumour

MCl was induced in a female mouse by subcutaneous (s.c.) injection of 3-methylcholanthrene (3MC) and the tumour, a fibrosarcoma was kept biofrozen in liquid nitrogen until use. The tumour which was passaged in the same strain up to ten transplant generations during experimentation, was found to be moderately immunogenic and tumour hosts are capable of rejecting up to 2×10^5 cells when reinoculated s.c. following excision of an implanted tumour.

Transfer assays

These were conducted essentially as previously described with the following modifications of splenic and LNC suspensions. Cells were incubated in a 5% CO_2 in air atmosphere in 6 in.-dia plastic Petri dishes in Eagles Minimum Essential Medium (MEM) containing 100 μg streptomycin and 100 i.u. benzylpenicillin/ml together with 10% heat-inactivated foetal Calf serum (FCS-MEM) at 37°C for 2 hr prior to further treatment. At this stage the splenic, but not LNC, were treated with 0.8% NH_4Cl solution for 5 min. They were diluted 20 times with FCS-MEM and spun at $250 \times g$ for 7 min. Both LNC and spleen cells were subjected to a total of five washes during which the cell suspensions were gently drawn in and forced out through a Pasteur pipette 20 times at each washing. This procedure was found to be essential in order to restore the effector cells from tumour bearing individuals to maximal reactive state as Currie and Basham [10] have demonstrated for *in vitro* cytotoxic assays. We have also found this to be equally relevant for *in vivo* assays using the transfer test which is shown in Table 1. Finally, lymphoid cells were suspended in MEM, without FCS, and diluted to the required concentration. Tumour cell suspensions were prepared as previously described and the two cell preparations mixed together in ratios of 5×10^4 tumour cells and 50–400 times as many lymphoid cells in a vol of 0.1 ml of MEM. A cell mixture sufficient for 5–10 inoculations was usually prepared and injections were given s.c. into the flank. Mice were observed and the appearance of tumours was assessed by palpation over a period of 30 days from the time of inoculation.

Assessment of DNA synthesis in lymphoid organs and in tumours

DNA synthesis in the lymphoid organs was taken as a measure of the stimiluation of cell proliferation assessed by the uptake of ^{125}I-deoxyuridine (IUDR) and described previously by Pelc, Harris and Galdwell [11] and by Asherson and Barnes [12]. Ten to twelve micro-

Table 1. *Effect of washing LNC from tumour-bearing mice on their anti-tumour activity shown in transfer assays*

Exp. No.	Group	Mean tumour diameter (mm) ± S.E.M. at intervals (days) after cell transfer	
		13	20/21
1. (a)	LNC washed × 5 and treated with NMS	3.18 ± 0.6	9.9 ± 1.3
	Washed again × 1		
(b)	LNC washed × 5 and treated with 25 day tumour-bearer serum	5.87 ± 0.69	9.2 ± 1.4
	Washed again × 1		
2. (a)	LNC washed × 6	4/5 just palpable	*2.5 ± 1.1
(b)	LNC washed × 5 day treated with 25 day tumour-bearer serum	4/5 palpable	10.5 ± 1.4
	Washed again × 1		

*2/5 mice did not develop tumours.

LNC = lymph node cells obtained from mice bearing MCl tumours for 10 and 25 days.

S.E.M. = standard error of the mean (exp. 1—10 mice per group: exp. 2—5 mice per group).

Each cell transfer inoculum contained 5×10^4 tumour cells together with 2.5×10^6 LNC and the mixture was injected s.c. into the flank in 0.1 ml MEM.

In exp. 1 group (a) differs significantly ($0.001 < P < 0.1$) from group (b) at 13 days. In exp. 2 group (a) differs significantly ($0.01 < P < 0.05$) from group (a) 20/21 days.

curie of IUDR (specific activity 108 mCi/mg; Radiochemical Centre Amersham) were injected i.p. into mice and after 17 hr the axillary and inguinal lymph nodes (ipsilateral and contralateral), spleen, thymus and tumour were carefully removed and the radioactivity counted in a well-type Packard γ counter without further processing of the tissues. Results were expressed as mean counts per minute corrected for background.

Enrichment of T cells

The method used was that described by Trizio and Cudkowsky [13] with the following modifications. Cells in FCS–MEM were incubated for 2 hr in plastic Petri dishes in 5% CO_2 in air following which they were recovered readily by gentle shaking leaving phagocytic cells attached. The cell suspension was concentrated and packed into nylon wool columns which were then incubated at 37°C in 5% CO_2 in air for 45 min. Eluted cells were concentrated and treated with 0.8% NH_4Cl for 5 min, diluted 20 times in FCS–MEM, and centrifuged. The washing procedure was repeated five times as described above. Enrichment of suspensions for T cells was demonstrated by indirect immunofluorescence using anti-θ serum followed by fluorescein conjugated swine anti-mouse γ globulin (SWAM–FITC) (Fraburg Limited, Maidenhead) when, around 90% of the cells were found to be labelled whereas over 90% of the cells did not label with SWAM–FITC) alone indicating the near absence of B cells in the preparations.

Statistical evaluation of results

The Students' *t*-test was performed to determine the statistical significance of the results in experiments on stimulation of DNA synthesis and also those on the assessment of tumour growth suppressive activity of lymphoid cells shown in Fig. 1 and in Table 1. Chi square test was utilized to determine the signficance of the difference between proportions of mice not developing tumours following cell transfer shown in Tables 2 and 3.

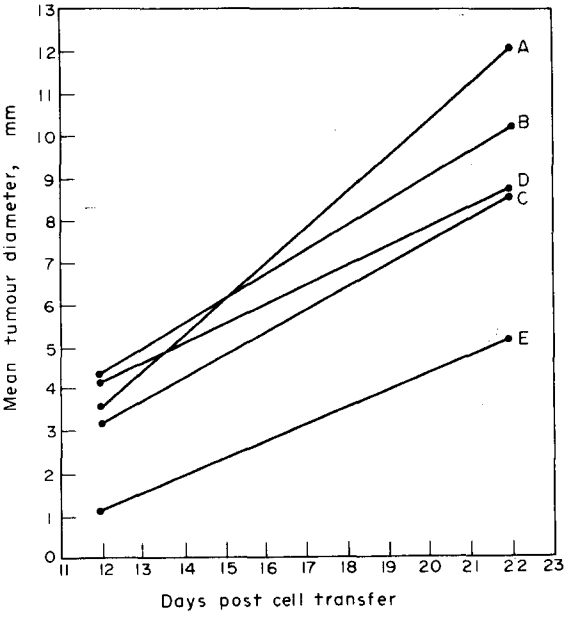

Fig. 1. Anti-tumour effect of tumour-bearer splenic T cell fraction in transfer assays. The rate of tumour growth in recipient mice following cell transfers is shown; 5×10^4 *MCl cells plus* 100–300 *times the number of splenic cells were inoculated into groups of* 5–6 *mice s.c. on the flank as follows:*

A—200 × *normal spleen cells.*
B—300 × *normal spleen cells.*
C—100 × 25 day *tumour bearer splenic T cells.*
D—200 × 25 day *tumour bearer splenic T cells.*
E—100 × 10 day *tumour bearer splenic T cells.*

Following differences between groups were significant: 12 days *after cell transfer, E vs C,* $0.01 < P < 0.02$; *(E vs D,* $0.05 < P < 0.1$*); 22 days after transfer, A vs E,* $0.001 < P < 0.01$.

RESULTS

Transfer assays

Groups of male BALB/c mice were implanted s.c. with 10^6 MCl cells and the tumours were

Table 2. Anti-tumour activity of LNC obtained from 10 *to* 14 day *and* 25 *to* 28 day *old tumour-bearing mice*

Exp. no.	Ratio of LNC to tumour cells	Days of tumour bearing in LNC donors		Normal controls inoculated with tumour cells only
		10–14	25–28	
1 (a)	50 : 1	7/9*	2/10	2/8
1 (b)	100 : 1	10/10	10/10	0/10
2	50 : 1	6/13	6/12	0/9

The proportion of the inoculated mice not developing tumours following cell transfer is indicated.
*Number of mice not developing tumours

Total number of mice in the group

In exp. 1 (a), difference between 10–14 and 25–28 day groups were significant ($\chi^2 = 4.23$, $0.02 < P < 0.05$).

Table 3. Anti-tumour activity of spleen cells obtained from 10 to 14 and 25 to 28 day old tumour bearing mice

Exp. No.	Ratio of spleen cells to tumour cells	Days of tumour bearing in spleen cell donors		Normal controls inoculated with tumour cells only
		10–14	25–28	
1 (a)	200:1	8/9*	1/10	0/9
1 (b)	400:1	10/10	9/10	1/9
2	200:1	7/9	3/9	1/9
Normal controls inoculated s.c. with 2×10^7 tumour-bearer spleen cells only			5/5	

The proportion of the inoculated mice not developing tumours following transfers is indicated.
*Number of mice not developing tumours / Total number of mice in the group
The following differences between 10–14 day and 25–28 day groups are significant, exp. 1 (a) ($\chi^2 = 8.87$, $0.001 < P < 0.01$); totals of exp. 1 (a) and exp. 2 ($\chi^2 = 11.9$, $0.001 < P < 0.01$).

allowed to grow for a period of 10–14 or 25–28 days. Nodes and spleens obtained from three or more mice were brought into single cell suspensions and were washed ×5 in MEM. Tumour cell suspensions were prepared from 10–14 day old tumours by trypsin disaggregation. The tumour and lymphoid cells were mixed together in the ratios of 1:50–1:400 and the mixtures were inoculated s.c. into groups of 5–10 normal mice so that each mouse received 5×10^4 tumour cells plus the appropriate number of lymphoid cells in 0.1 ml of MEM. The appearance of tumours was observed over a period of 30 days.

Comparison of the anti-tumour activity of the lymph node lymphocytes at different stages of tumour bearing

Table 2 shows that LNC taken from 10–14 day tumour bearers were markedly suppressive to the tumour cells. In two transfer assays, a total of 13/22 mice remained tumour free following transfer of cells at a LNC to tumour cell ratio of 50:1 compared with 2/17 of the control group inoculated with tumour cells alone. When the cell ratio was increased to 100:1 total suppression of tumour formation was observed in the cell transfer recipients of the test group whereas all 10 controls inoculated with tumour cells alone, developed tumours. The addition of 200 or 300 times as many normal spleen cells into the tumour cell inoculum had no influence on the outgrowth of tumours (Fig. 1).

Comparison of the tumour suppressive activity of LNC from the 10–14 day stage of tumour bearing with those at 25–28 days showed no significant difference when the LNC to tumour cell ratio utilized in transfer assays was 100:1. Total suppression of tumour outgrowth was obtained under these conditions. However, this cell ratio may not be sensitive enough to detect more subtle differences. Thus in one of two transfer assays conducted with a LNC to tumour cell ratio of 50:1, 7/9 mice did not develop tumours when the LNC were derived from the 10–14 day tumour bearers in contrast to 2/10 when the cells were taken from 25–28 day tumour-bearing mice. This difference was found to be significant ($0.01 < P < 0.02$). However, in a second transfer assay, tumour rejection was observed in 6/13 and 6/12 mice, respectively, in the two groups. This variability of results obtained at this lower cell ratio may indicate the limit of sensitivity for the technique under these experimental conditions. Nevertheless, the results obtained from the first experiment indicate that some loss of anti-tumour activity has taken place in the LNC population at the 25–28 day stage of tumour bearing.

Comparison of anti-tumour activity of splenic lymphocytes at different stages of tumour bearing

In experiments using 10–14 day old tumour bearer spleens, tumour suppression was very significant when the spleen cell to tumour cell ratio was 200:1, as compared with either the effects of 25–28 day spleen cells or with normal controls inoculated with tumour cells alone. In two transfer assays, 8/9 and 7/9 mice receiving the cells from the 10–14 day stage, as compared

to 1/10 and 3/9 mice receiving cells from the 25–28 day stage of tumour bearing, respectively, did not develop tumours following cell transfer. The difference 8/9 vs 1/10 in the first assay was found to be significant by the Chi-square test ($0.01 < P < 0.001$) as was the difference between the totals 15/18 vs 4/19 from the two experiments ($P = 0.001$). Controls which received the same number of tumour cells as were present in the cell transfers did not develop tumour only in 1/18 of the inoculated mice. Cell transfers using a spleen to tumour cell ratio of 400:1 led to total suppression of tumour outgrowth in both the 10–14 and 25–28 day tumour-bearer spleen cell recipients. Inoculation of as many as 20×10^6 spleen cells from 28 day tumour-bearers produced no tumours in any of five inoculated mice demonstrating the absence of metastatic cells capable of tumour formation.

Effector cell activity of column-enriched spleen (T) cells from tumour-bearing mice

Cell transfer assays were carried out using 10 and 25 day tumour-bearer splenic T cells with tumour to spleen cell ratios ranging from 1:100 to 1:300. These column purified suspensions contained less than 10% B cells as detected by SWAM-FITC. Each recipient was inoculated s.c. with 5×10^4 tumour cells together with the appropriate number of splenic T cells and the cell mixtures transferred into groups of 5–6 mice. Figure 1 shows that when $\times 100$ as many splenic T cells as tumour cells were used 2/5 recipients of the 10 day tumour bearer splenic T cells did not develop tumours compared with 0/5 after 12 days and 1/5 after 22 days in the recipients of 25 day splenic T cells. There was also a significant difference ($0.01 < P < 0.02$) in the growth rates of the resulting tumours in the two groups detected as tumour volume, 12 days after cell transfers, the 10 day spleen T cell recipient groups showing a marked reduction in tumour growth compared to the 25 day T cell recipients. The use of $\times 200$ the 25 day splenic T cells as tumour cells did not cause a significant difference. Control groups which received $\times 200$ or $\times 300$ the normal spleen cells as tumour cells did not lead to suppression of tumour formation and there was no significant difference in their growth rates compared to the 25 day spleen cell recipients.

Stimulation of DNA synthesis in lymphoid organs of tumour-bearing mice

A comparison was made of the rate of stimulation of DNA synthesis in the lymphoid organs of mice bearing tumours for 14 or 25 days. Two groups of four mice were injected s.c. with 10^6 MC1 cells, 11 days apart, so that at the time of testing they had borne tumours for 14 or 25 days. Both groups of mice and a third group of four normal controls, were injected i.p. with 12 μCi of IUDR and 17 hr later the ipsilateral and contralateral lymph nodes, spleen, thymus and tumours were removed and the radioactivity measured. Results were expressed as counts/min (cpm or as a stimulation index (SI).

Table 4 shows that DNA synthesis in the lymph nodes of mice bearing tumours for 25 days

Table 4. *Stimulation of DNA synthesis in lymphoid organs of mice* 14 *and* 25 days *after tumour initiation*

No. of days following s.c. implantation of 10^6 MCl cells	ILN		CLN		Spleen		Thymus		Tumour	
	Counts/min ±S.E.M.	SI	Counts/min ±S.E.M.	SI	counts/min ±S.E.M.	SI	counts/min ±S.E.M.	SI	counts/min ±S.E.M.	
14	3179±529	12.2	3789±1408	14.6	34318±2971	2.7	2525±326	1.4	25657±3629	
25	566±73.5	2.2	363±25.6	1.4	48519±3747	3.8	1147±157.7	0.6	47074±5533	
Normal Controls	*261±26.8		*259±23		12765±3927		1800±193		—	

The mice were injected i.p. with 12 μCi IUDR and the tissues removed 17 hr later. The activity incorporated is indicated as counts/min ± standard error of the mean (S.E.M.).

$$\text{SI} = \text{Stimulation Index} = \frac{\text{Mean counts/min in test group}}{\text{Mean counts/min in normal controls}}.$$

ILN = ipsilateral lymph nodes = (axillary and inguinal nodes on the same side as the tumour).

CLN = contralateral lymph nodes = (axillary and inguinal nodes on the opposite side to the tumour).

*These figures were obtained from axillary and inguinal lymph nodes from either the right or left flank.

The differences in counts/min between 14 and 25 day experimental groups are significant for following tissues, ILN ($0.001 < P < 0.01$); CLN ($P = 0.05$); spleen ($0.02 < P < 0.05$); thymus ($0.001 < P < 0.01$); tumour ($0.001 < P < 0.01$).

Table 5. Resistance to subsequent growth of tumour in mice pre-immunized to the same or different tumour

Immunizing tumour	Challenge tumour	Proportion of mice rejecting challenge tumour
MC1	MC1	*4/4
	MC5	0/4
MC5	MC1	0/4
	MC5	3/4
EAT	MC1	0/4

*Number of mice rejecting tumour / Total number of mice in the group

was markedly depressed compared to the level at 14 days. Significant differences were seen between the cpm and SI of the ipsilateral nodes ($0.001 < P < 0.01$) and between that of contralateral nodes ($P = 0.05$). These are also shown in their stimulation indices. In the thymus the decrease of DNA synthesis at 25 days was signficantly below the level of 14 days ($0.001 < P < 0.01$). In contrast DNA synthesis in the spleen was signficantly elevated at 25 days ($0.02 < P < 0.05$) as compared to that at 14 days and similarly DNA synthesis in the tumour was also markedly increased reflecting an increase in tumour mass ($0.001 < P < 0.01$).

The role of "specific" tumour associated antigens

Tumours were initiated in groups of mice as follows: 8 mice implanted s.c. with MC1, 8 mice with MC5 and another 5 with Ehrlic ascites tumour (EAT). When the tumours reached 0.5–1 cm dia by 10–14 days, they were surgically excised and all groups were given a rest period of 14–18 days, prior to tumour challenge as follows. Four mice immunized with MC1 were challenged with 5×10^4 MC1 cells and four were challenged with 5×10^4 MC5 cells. The group immunized with the MC5 was again split into two further groups and these were challenged as above with either MC1 or MC5. The mice immunized to the EAT were challenged with 5×10^4 MC1 cells only.

Table 5 shows that mice immunized to MC1 rejected MC1 cells while those immunized to MC5 or EAT developed tumours. Conversely, mice immunized to MC5 failed to suppress MC1 although MC5 immune mice rejected that tumour.

These results indicate clearly that the MC1 tumour contains specific tumour associated antigens which are neither shared with MC5, another syngeneic tumour, nor with the EAT, which probably contains several alloantigenic specificities.

DISCUSSION

The experiments described here show that the total anti-tumour activity generated by the lymph nodes and spleen at 10–14 days following tumour initiation was markedly stronger than at 25–28 days.

Proportionally, the detected tumour suppressive activity in the washed LNC population changed but little, under the experimental conditions employed, despite the very significant decrease of DNA synthesis and cell proliferation in nodes at the latter stage. This finding contrasts with the observations in the spleen. In the latter the very significant increase of stimulation of DNA synthesis observed at 25 days was also accompanied by a hyperplasia and a marked increase in the total number of cells in these lymphoid organs whilst the anti-tumour activity per cell decreased significantly. The decreased tumour suppressive activity of the spleen cells could have been the result of dilution of the effector cell population by the increased proliferation of non-specific cell clones, or cell clones having functions other than tumour suppressive activity. It is interesting to note in this context that Konda, Nakao and Smith [14] showed that both T and B splenic cell populations increased numerically as well as in their functional capacity to respond to a variety of non-tumour antigens during tumour-bearing. It appears therefore, that increase of cell clones with different functional capabilities can occur during the "suppression" of stimulation of cells with anti-tumour activity in lymph nodes and in the spleen. The decrease in anti-tumour activity of the spleen cell population was associated with T cells since transfer assays conducted with a nylon wool column-enriched T cell fraction was markedly less effective in tumour suppression at the latter stage of tumour bearing. This suggests a retarded proliferation of T cells with anti-tumour activity and an increase in the ratio of non-cytotoxic to cytotoxic T cells.

The findings reported here support our previous observations of the suppression of the immune response to MC-induced tumours [6, 7, 9] which showed that the anti-tumour response is suppressed or modulated by factors acting at the level of sensitization of the lymphocytes in addition to those factors involved in the blocking mechanisms. There is increasing evidence that suppressor mechanisms are in-

volved in the modulation of the immune response to non-neoplastic tissues [15, 16] as well as to tumours. Treves, Cohen and Feldman [17] reported a suppressed ability of tumour rejection or enhancement of tumour growth in animals treated with tumour-bearer spleen cell culture filtrates. In an interesting recent report Fujimoto, Greene and Sehon [18, 19] observed that suppressor cells can be found in spleen, nodes, thymus and bone marrow of tumour-bearers and that treatment of tumour transplant recipients with appropriate doses of such cells caused suppression of tumour rejection. Chandradasa [6, 7] had previously observed that intraperitonial inoculation of 200×10^6 spleen cells from tumour-bearing mice to other mice, at the time of tumour implantation, caused tumour enhancement and an increase of metastatic growths in the recipients. The observations reported here provide further evidence for the involvement of a defect of sensitization and proliferation of effector cells and the possible association of suppressor mechanisms in the modulation of the immune response in tumour-bearers and the facilitation of tumour growth.

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Amputation and Doxorubicin for Treatment of Canine and Feline Osteogenic Sarcoma*

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Abstract—*Fourteen dogs and* 3 *cats with naturally occurring oesteogenic sarcoma of the appendicular skeleton were treated with adjuvant chemotherapy using doxorubican* HCl *following treatment of the primary tumor by amputation or disarticulation. Doxorubican* HCl *was given intravenously every* 21 days *at a dosage of* 30 mg/m^2 *body surface area commencing* 3 *weeks after amputation, and continuing for* 6 *months or until metastatic disease was recognized radiographically. The median duration of clinical signs prior to surgical treatment for* 14 *dogs was* 51 days (*mean,* 54 days; *range,* 7–127 days). *The median time interval between surgery and onset of radiographically evident metastatic disease for* 9 *dogs was* 73 days (*range* 50–160 days). *Median post-operative survival for* 14 *dogs was* 104 days (*range* 52–368 days); 35% *were alive* 6 *months post-operatively,* 21% *were still alive at* 9 *months and* 14% *survived more than one year. Times to onset of metastatic disease and survival times were calculated from day of surgical treatment.*

For 3 *cats duration of preoperative clinical signs were* 24, 70 *and* 95 days; *intervals prior to metastatic disease for* 2 *cats were* 133 *and* 274 days; *and survival times were* 143, 180 *and* 414 days.

INTRODUCTION

With the possible exception of acute leukemia, most of the screening and secondary evaluation of potential anticancer agents is done in animal tumor systems that lack an objectively demonstrated correlation with the drug response of any specific type of human neoplasm. The search for better animal tumor models continues and the Ridgway osteogenic sarcoma is a valuable addition for the experimental chemotherapist [1]. The clinical, radiologic and pathologic features of spontaneous canine and feline osteosarcoma have been previously described [2–5], and it has been suggested that the dog is a useful model for study of osteosarcoma in man [6].

Despite the advances made in surgical and radiotherapeutic techniques, the treatment of dogs with osteogenic sarcoma results in relatively few survivors. Because pet owners frequently request euthanasia for their animal upon recognition of malignant disease, survival times for untreated (control) dogs with osteogenic sarcoma are not available. However, an historical control population of 65 dogs with naturally occurring oesteogenic sarcoma of the appendicular skeleton treated by surgery alone was reported [2]. In that study, end-results were as follows: the median post-operative survival was 18 weeks, with a range of 3 to greater than 578 weeks. Twenty-six percent were alive 6 months post-operatively, 13.8% were still alive at 9 months and 10.7% survived more than one year. The common cause for therapeutic failures was pulmonary metastasis.

Other methods used to control primary or metastatic disease in the dog have included irradiation, chemotherapy, immunotherapy, or combinations of those treatment modalities. Response of canine osteogenic sarcoma to irradiation has been discouraging. In one study, 18 animals were treated with conventional radiotherapy (X-rays or ^{60}Co) or fast neutrons (^{2}N). While cessation of tumor growth was apparent in some cases, osteogenic sarcoma was considered non-responsive to irradiation [7]. Thirty-one small animals in another study with osteogenic sarcoma were treated to an average of 5000 rad by various forms of irradiation; the percentage cure was zero [8]. Prophylactic X-irradiation of lungs did not inhibit pulmonary metastasis in 22 other dogs with osteogenic sarcoma when combined with primary therapy using amputation or X-irradiation [9].

Few chemotherapeutic studies for management of osteogenic sarcoma in the dog have been

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reported [10–13]. Small numbers of animals treated and wide range of treatment modalities used makes comparison of data difficult. The use of combinations of anticancer drugs following amputation in 8 dogs reported in one study, however, resulted in median post-operative survival time of 10.5 months (mean, 12.3 months: range, 3–23 months) suggesting possible therapeutic advantage of systemic (adjuvant) chemotherapy following primary (surgical) therapy [13].

Immune therapy has been investigated preliminarily for treatment of canine osteogenic sarcoma [14]. Freeze-dried BCG vaccine was given intravenously to 6 dogs following amputation; median survival time was 52 weeks (mean, 46 weeks; range, 16–65 weeks) compared to 5 non-matched control dogs treated by amputation alone surviving 6–17 weeks (mean, 11 weeks).

Primary bone cancer has been reported infrequently in the cat. In a report of 15 cats with osteogenic sarcoma, the neoplasm tended to occur in the hind limbs of aged, female, domestic, short-hair cats [3]. For 5 cats treated by amputation of the affected limb, one cat developed metastatic disease 5 months post-operatively, while the other 4 cats were alive and non-metastatic 26 months after surgery.

MATERIAL AND METHODS

Animals and treatment

Fourteen privately owned dogs and 3 cats with spontaneously occurring osteogenic sarcoma of the appendicular skeleton were referred to the University of California, Veterinary Medical Teaching Hospital. Presumptive diagnosis of osteogenic sarcoma was based on clinical and radiographic examinations; definitive diagnosis was made in all cases by histopathology. As in man [15], osteosarcoma in the dog [4] is a tumor composed of malignant cells that produce osteoid.

Primary therapy used in all cases was amputation—a scapulohumeral disarticulation for neoplasms of the radius, ulna and distal humerus; mid-shaft femoral amputation for neoplasms of the distal tibia; coxofemoral disarticulation for neoplasms of the proximal tibial or femur. Block ostectomy was used in one dog (No. 4) for a neoplasm of the distal ulna. Prior to the therapy, the following studies were performed: radiographic examination of the tumor site and of the thorax for metastatic disease, complete blood count, urinalysis and a 12- channel biochemical profile. Excluded from study were animals with radiographically demonstrable metastatic disease, animals with concurrent medical problems which would preclude use of anticancer drugs, and those giant breed dogs which could not tolerate amputation because of severe concurrent orthopedic problems (e.g. hip dysplasia). Animals studied received chemotherapy using doxorubicin HCl (adriamycin) at a dosage of 30 mg/m^2 of body surface area starting 3 weeks post-operatively to allow surgical wound healing. Drug therapy was given intravenously every 21 days for 24 weeks. During the course of therapy, thoracic radiographs were obtained at 6 week intervals and hematologic and biochemical tests were monitored at 2–6 week intervals. Drug therapy was discontinued upon radiographic recognition of metastatic disease.

RESULTS

Table 1 lists breed, age, sex, site of primary neoplasm, duration of presurgical signs, time to onset of radiographically demonstrable pulmonary metastasis, total survival time and cause of death in 14 dogs and 3 cats with osteogenic sarcoma of the appendicular skeleton treated by surgery followed by systemic adjuvant chemotherapy. The 14 dogs were all large or giant breeds, known to be subject to excess risk of primary bone sarcoma [16]. Times given for onset of metastatic disease and survival are calculated from the day of amputation or disarticulation. The median duration of clinical signs prior to definitive therapy for the 14 dogs was 51 days (mean, 54 days; range, 7–127 days). The median time to onset of radiographically apparent metastatic disease for 9 dogs that developed metastasis was 73 days (range, 50–160 days). Two of the 3 cats developed pulmonary metastasis 133 and 274 days post-operatively; prolonged survival for one cat (No. 17) following recognition of metastatic disease appears inordinate. The third cat did not have post-mortem evidence of metastatic disease 180 days post-operatively when euthanatized because of intercurrent gastro-intestinal disease.

Median post-operative survival for 14 dogs was 104 days (range, 52–368 days). Two of the 14 dogs are still alive at time of writing. As in other clinical veterinary trials, survival is often compromised by elective euthanasia by pet owners. Two dogs in this study (Nos. 6 and 8), euthanatized soon after recognition of pulmonary metastasis were not clinically ill at time of death; all other dogs showed signs of systemic illness at the time of euthanasia. Excluding those 2 dogs from the survival data provides median post-operative survival time of 117 days.

Survival times for 3 cats were 143, 180 and 414 days post-operatively.

Causes for death are listed; 13 of the 14 animals that died during the study were necropsied. Unusual metastatic patterns were observed in several dogs. Dog No. 2 had metastasis to the right atrioventricular valve in addition to pulmonary metastasis; dogs No. 5 and 11 developed renal metastases in addition to pulmonary lesions. Dog No. 14 developed severe clinically apparent multiple skeletal metastases accompanied by few small pulmonary lesions that were not visualized radiographically.

Other lesions observed at post-mortem examinations were as follows: dogs No. 1 and 12 had histological evidence of myofibrillar atrophy and acute tubular nephrosis was identified in dogs 1, 4 and 5. Dog No. 2 had hypertrophic osteopathy. Dog No. 7 had diffuse interstitial pneumonitis and fibrosis. Cat No. 16 had both metastatic osteogenic sarcoma and malignant melanoma in lungs at necropsy. The primary site for the melanoma was not determined, but was presumably related to an ocular lesion treated two years earlier by enucleation.

DISCUSSION

The results which have been achieved in the treatment of osteogenic sarcoma by immediate surgery have been disappointing in man and dogs. Although local control can generally be achieved in primary lesions of the extremity, pulmonary metastasis and death within 12–18 months occur in over 80% of human patients with osteogenic sarcoma [17], and 90% of dogs with osteogenic sarcoma are dead 12 months post-operatively [2]. In dogs, less than 10% show radiographic evidence of pulmonary metastasis at the time of initial examination [9]. However, the subsequent "natural" course of the disease indicates that hematogenous pulmonary micrometastases are present in the majority of dogs at the time of diagnosis, or that tumor cells are released into the circulation during amputation. The problem of how to inhibit division of these malignant cells in the lung before they become apparent radiographically or clinically is a major goal in cancer therapy research; dogs with osteogenic sarcoma must be considered as having systemic disease when the diagnosis is first made and the treatment program planned accordingly.

During the past few years, several publications have indicated that the prognosis in osteogenic sarcoma in man is improving significantly as the result of adjuvant chemotherapy [18–20]. Early 1970's, a relatively new drug known as doxorubicin became available for clinical studies, and the effectiveness of doxorubicin in the treatment of metastatic osteosarcoma was soon apparent. Subsequent activity of the drug demonstrated in an adjuvant (prophylactic) setting prompted our interest in doxorubicin for treatment of canine and feline osteogenic sarcoma. For adjuvant chemotherapy to be effective, the primary lesion must be controlled [21]. Disarticulation or transmedullary amputation will usually insure ablation of the primary tumor in canine osteogenic sarcoma [2]; the only dog (No. 4) that developed local recurrence in this study was treated by block ostectomy of the distal ulna instead of radical amputation. While surgical resection will control primary disease, it appears unlikely from published data [7, 8] that radiotherapy alone will be similarly effective.

Dosage of doxorubicin and route of administration for dogs and cats in this study was based on published toxicity data in the dog [22, 23] and cat [24] and experience treating animals in our clinic [13]. As in man, doxorubicin in the dog and cat behaves as an inhibitor of cellular reproduction; damage to actively proliferating tissues is most prevalent and toxic manifestations reported and encountered in our experience include enterocolitis, alopecia, melanosis, hyperesthesia, shock-like syndrome and depression of hematopoiesis. Functional or morphologic damage to the heart was not encountered in the drug toxicity study [22]; however, focal myofibrillar atrophy diagnosed histologically in two dogs in this study may have resulted from doxorubicin toxicity. Similarly, nephrosis has not been reported in dogs or man as a manifestation of doxorubicin toxicity and development of nephrosis in 3 dogs reported herein was unexpected. While the possibility of pre-existing renal disease cannot be excluded, normal blood urea nitrogen values and urinalyses were obtained in all dogs prior to doxorubicin chemotherapy. Severe tubular nephrosis has been demonstrated in rabbits following doxorubicin treatment [22].

While the prognosis for a dog with osteogenic sarcoma is poor, the disease is not uniformly fatal following surgery alone for treatment of primary disease [2]. Prospective adjuvant chemotherapy trials using small numbers of animals is compromised by the contention that animals cured may have received unnecessary adjuvant chemotherapy. Additional difficulties encountered in a clinical veterinary setting include reluctance of pet owners to allow aggressive chemotherapy because of toxic side effects and costs of treatment. Clinical benefit from doxorubicin in man is correlated with the consistency of

Table 1. *Results of surgical treatment and adjuvant chemotherapy using doxorubicin in* 14 *dogs and* 3 *cats with osteogenic sarcoma*

Dog No.	Breed	Age (yr)	Sex	Site	Duration of presurgical clinical signs (days)	Time to onset of metastatic disease (days)	Survival time (days)	Cause of death
1.	Saint Bernard	6	F	Right distal tibia	14	160	230	Euthanatized because of cachexia. Post-mortem evidence of nephrosis and myofibrillar atrophy
2.	Irish Setter	2	M	Right proximal tibia	25	73	73	Euthanatized because of hypertrophic osteopathy. Metastasis to lungs, and right atrioventricular valve
3.	Irish Wolfhound	7	M	Left proximal tibia	7	NM*	365†	No evidence of metastatic disease
4.	German Shepherd	10	F	Left distal ulna	77	NM	386	Died from acute tubular nephrosis. No evidence of metastasis at post-mortem. Local recurrence at ostectomy site
5.	Great Pyrenees	7	F	Right proximal humerus	60	67	75	Euthanatized because of cachexia. Metastasis to lung and kidney. Tubular nephrosis evident histologically
6.	Saint Bernard	8	M	Right distal radius	24	106	106	Euthanatized at recognition of metastasis. Dog not systemically ill
7.	Irish Setter	4	M	Left distal ulna	43	NM	265	Died from diffuse interstitial pneumonitis and fibrosis

8.	Rhodesian Ridgeback	5	M	Left proximal humerus	58	60	83	Euthanatized at recognition of metastasis. Dog not systemically ill
9.	Great Dane	5	M	Right distal radius	26	NM	300†	No evidence of metastatic disease
10.	Great Pyrenees	5	M	Right distal tibia	48	109	131	Euthanatized because of cachexia. No post-mortem examination
11.	Doberman Pinscher	9	M	Right proximal humerus	127	50	52	Euthanatized because of metastasis and intercurrent cervical disc prolapse. Metastasis to lung, liver, kidney
12.	Great Pyrenees	3	M	Left distal radius	75	NM	80	Died acutely; focal myofibrillar atrophy demonstrated histologically
13.	Airedale Terrier	9	M	Left distal radius	120	60	102	Euthanatized because of cachexia. Metastasis to lungs
14.	German Shepherd	9	M	Right proximal humerus	54	92	92	Euthanatized because of multiple metastases to bone and lungs
15.	Domestic Short-haired cat	10	F	Left distal femur	95	NM	180	Euthanatized because of intercurrent gastrointestinal disease
16.	Domestic Long-haired cat	7	M	Right proximal tibia	70	133	143	Euthanatized because of cachexia. Metastasis to lungs
17.	Domestic Long-haired cat	12	M	Left distal femur	24	274	436†	Still alive despite metastatic disease

*NM = Non-metastatic.
†Animal still alive at time of writing.

myelosuppression following administration of drug [25] and neoplasms such as osteogenic sarcoma treated with inadequate doses are likely to remain resistant to treatment [26]. Dose-limiting myelosuppression was infrequently encountered in dogs treated in this study, suggesting that the dosage of drug used may have been clinically ineffectual.

End-results obtained for treatment of osteogenic sarcoma using amputation and doxorubicin in this study is unimpressive. Using survival as a measure of response to therapy, the median post-operative survival times are less than those reported for surgery alone [2] and less than those reported for surgery followed by combination chemotherapy [13]. However, 5/14 dogs (35%) were alive 6 months post-operatively, 3/14 (21%) were still alive at 9 months and 2/14 (14%) survived for more than one year which is not worse than the historical group treated by surgery alone. Risks of doxorubicin treatment, however, may be disproportionate to the expected benefits and unexpected toxicoses suggest that additional drug evaluation in normal dogs is warranted.

Relevant criteria for evaluating end-results of treatment of the animal cancer patient include time to onset of metastatic disease (disease-free interval), and total survival time. Elective euthanasia at recognition of metastatic disease in some cases jeopardizes survival data and suggests that disease-free interval following primary treatment is the most relevant criteria of response to therapy in clinical veterinary oncology.

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Serum Alpha-fetoprotein in Experimental Hepatocellular Carcinomas in Rats*

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Abstract—*The present study has been carried out mainly to study the sequential changes of serum alpha-fetoprotein (AFP) levels with the evolution of experimental hepatocellular carcinoma (HCC) and to correlate with the histology of the tumors. Rats fed with* 0.06% *of azodyes developed HCC by* 8–24 *months and on the basis of their capacity to synthesize AFP, the animals can be divided into two distinct major groups– "quick responders" and "slow responders". In the first group the animals developed tumors by* 8–10 *months and mostly of poorly differentiated type with high serum AFP levels. The slow responders developed tumors only after* 16 *months and they were mostly of well differentiated with comparatively low AFP values. Both the groups showed biphasic response of AFP during the evolution of HCC. The animals in aflatoxin B*1 *group did not show AFP levels more than* 2μg/ml.

INTRODUCTION

ALPHA-FETOPROTEIN is a feto specific serum globulin synthesised by fetal liver and secreted into the serum during embryonal life of mammalian species. Abelev *et al.* [1], for the first time reported the presence of AFP in blood of mice bearing transplantable hepatomas. Reappearance of fetal antigens is now recognised as common phenomenon during oncogenesis. Elevated levels of serum AFP during hepatic carcinogenesis has subsequently been reported in different species of animals including man [2, 3]. Besides its appearance in rats with chemically-induced HCC its early elevation has also been demonstrated within weeks after the administration of chemical carcinogens [4]. However, certain chemical carcinogens like aflatoxin induced HCC which are not associated with significant rise of AFP [5].

The technique of agar gel immunodiffusion has been used for the estimation of serum AFP, but the sensitivity is relatively low. Among the more sophisticated methods for the early detection of HCC, radioimmunoassay (RIA) happens to be most sensitive one. In fact the use of RIA has revealed that even normal individuals and animals have a minimum quantity of AFP in circulation [6].

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The present investigation was undertaken to study the serum AFP levels using RIA during the evolution of liver cancer using different types of chemical carcinogens and to correlate the morphological type of lesions with AFP synthesis.

MATERIAL AND METHODS

Wistar strain rats of both sexes weighing 90–120 g were kept in groups of four in wire mesh cages and maintained on laboratory diet. The carcinogens used were as follows:
Batch 1: (50 animals) was fed with 0.06% of 4-dimethyl-aminoazobenzene (DAB). Batch 2 (52 animals) with same dose of 3-methyl-4-dimethylaminoazobenzene (3-Me-DAB) and batch 3 (10 animals) was administered aflaxtoxin B1 (gift from E. K. Weisburger, National Cancer Institute, Bethesda, Maryland, U.S.A.) orally 25 μg in normal saline for 5 consecutive days for 8 weeks [7]. Control animals were maintained for each group. The azodye containing diet was fed to the rats for 6 months and then they were kept on normal diet. Due to lack of azodyes during the experimental period for some time, feeding with the experimental diet was discontinued after 10 weeks in 15 animals in each group and they were observed for 24 months for the development of HCC. The animals were sacrificed after the end of the experiment.

The animals were either sacrificed or biopsied at monthly intervals and tissues processed after

fixing in fresh Carnoy's fluid for 24 hr. Sections were cut at 4 μm, stained by H&E and Toluidine blue. Blood was collected at monthly interval by puncturing the occular sinus, serum was separated and stored at −20°C until further use.

Radioimmunoassay. Quantitative estimation of serum AFP was done by RIA using double antibody technique [8]. Pure rat AFP was kindly supplied by International Agency for Research on Cancer, Lyon, France. Monospecific antibody raised in sheep was kindly supplied by Dr. Helena Phiko.* Antisheep gamma-globulin was raised in rabbit in our laboratory. Purified rat AFP was iodinated by chloramine-T method [9]. The radioimmunoassay was carried out as shown below:—

PBS, 0.05M, p.H. 7.0 (0.1% gel)	0.4 ml
Serum or standard	0.1 ml
Labeled AFP (10,000 counts per 100 sec)	0.1 ml
Sheep antirat AFP (1 in 4000 dilution)	0.1 ml
Normal sheep serum (1 in 100 dilution)	0.1 ml
Mixed, incubated at 4°C for 24 hr	
Rabbit antisheep gamma-globulin (1 in 3 dilution)	0.1 ml
Mixed, incubated at 4°C for 24 hr	
2000 rev/min for 30 min	
Counted the precipitate	

The antibody dilution binding at least 20% of the precipitable counts was selected for the inhibition test. Standard inhibition curve was obtained by diluting pure AFP by two fold dilution ranging from 1000 to 1 ng and standard curve was plotted. Serum samples were tested in duplicate and the values were read against the standard curve (Fig. 1).

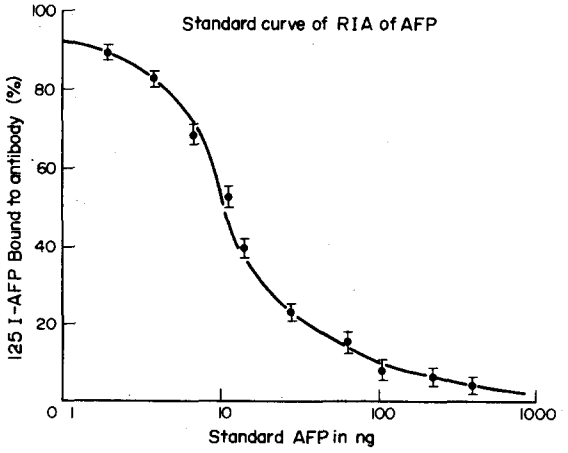

Fig. 1. Standard curve of radioimmunoassay of rat AFP.

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From the standard curve obtained under the condition of the assay used, quantitation of purified rat AFP above 1 ng/0.1 ml could be reproducibly demonstrated. Hence a quantity of 10 ng of AFP per ml of serum sample could be detected with confidence. A set of dilution of known serum sample containing AFP and a set of standard AFP were used for calibration. The curves plotted on the same graph showed parallelism indicating specificity. The assay precision as determined by the coefficient of variation of inter and intra assay of reference sample did not exceed 10%.

Synthesis of AFP in the tumor tissue was demonstrated by immunoperoxidase technique [10] and the tissues were processed according to the Sainte-Marie's method [11].

RESULTS

The serum AFP levels of normal rats were 10–80 ng/ml, the average being 49 ng/ml. Males showed higher values (57 ± 9.4 ng/ml) than females (41 ± 14.1 ng/ml) and also the incidence of HCC in males was significantly higher.

A biphasic reaction of serum AFP levels was observed in most of the animals during the evolution of HCC with azodye carcinogens. The rats that were fed with diet containing 0.06% of azodyes for 6 months developed HCC during 8–24 months of observation. The serum AFP levels began to rise in the first month and increased to attain a peak between second and third month with the mean of 4 μg/ml. By the third month a clear suppression of AFP synthesis could be appreciated. From the fourth month onwards a majority of the animals showed subsequent progressive rise in AFP with the appearance of HCC. The sequential changes in AFP levels are depicted in Fig. 2.

Animals that showed the maximum AFP levels during the first 3 months were the earliest to develop HCC by 8–10 months (Group A). The subsequent serum AFP levels of these animals ranged from 250 to 1100 μg/ml. Two animals, which showed the serum AFP levels 7 μg/ml. during the first peak period, developed HCC as early as 8th month, showing a maximum AFP levels of 1100 μg/ml. Animals which showed gradual rise of AFP levels from fifth month onwards developed HCC by 16–24 months and the levels were less than 15 μg/ml (Group B). Three animals (Group C) which failed to show any rise of serum AFP during the first peak period, yet developed HCC by 16–24 months and the AFP levels were low as

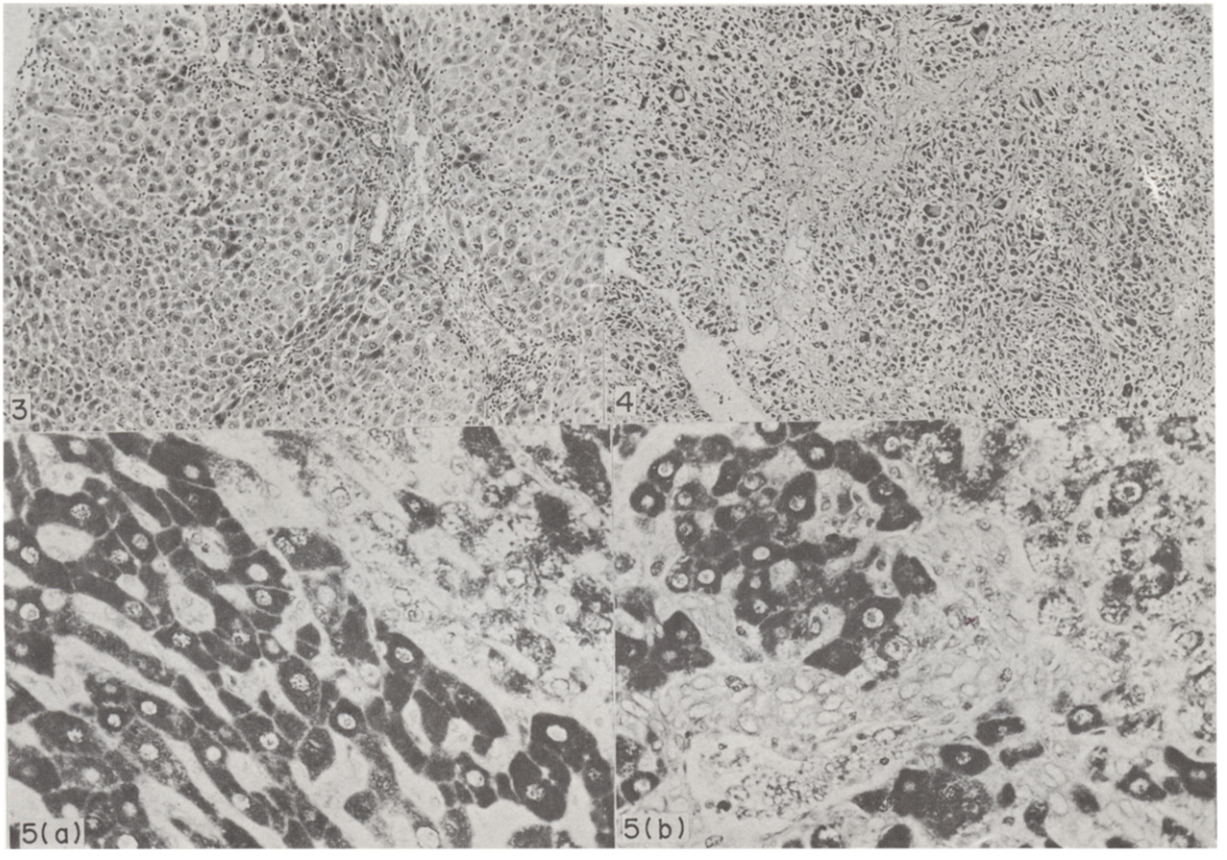

Fig. 3. *Hepatocellular carcinoma of grade II in DAB carcinogenesis.* H and E × 110.

Fig. 4. *Hepatocellular carcinoma of grade IV (anaplastic type) showing spindle shaped cells with numerous giant cells.* H and E × 110.

Fig. 5. (*a*) *Tumor showing lack of AFP in highly ill differentiated cells (Top right). The adjoining poorly differentiated cells show intense reaction. Immunoperoxidase* × 440.
(*b*) *Random distribution of AFP synthesis by tumor cells lying singly or in clusters with intermixing AFP negative cells. Immunoperoxidase* × 440.

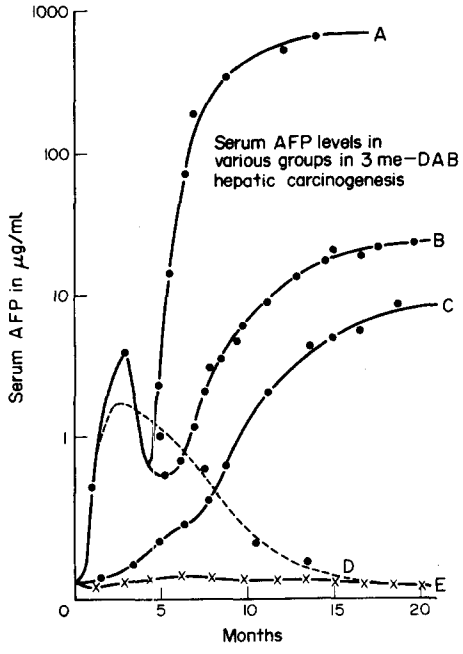

Fig. 2. Serum AFP levels in 3-Me-DAB carcinogenesis. Group A quick responders: groups B and C slow responders. Group D showed only primary reaction and group E no rise of serum AFP levels. No tumors developed in groups D and E.

compared to animals which showed biphasic response. Four animals (Group D) which showed initial rise of AFP during the first three months did not develop HCC and there was no subsequent rise of AFP. Four animals (Group E) did not show any rise of AFP during the whole period of experiments. Since the animals were being sacrificed at regular intervals to study sequential changes, it was not possible to document either the percentage of animals that developed HCC nor the precise percentage of animals showing absence of a second peak of AFP synthesis. However, the results of AFP levels of some of the animals have been depicted in the graph. Fig. 2.

Twelve animals (7 in 3-Me-DAB and 5 in 4-DAB group), which were fed with experimental diet for $2\frac{1}{2}$ months only, also developed HCC after 20 months. None of the animals in aflatoxin B1 group showed rise of AFP levels more than 1–2 μg/ml and 2 animals developed tumors after 26 months.

Histology

Tumors were classified into four grades. Grade I, well differentiated; grade II (Fig. 3), moderately differentiated; grade III, poorly differentiated and grade IV, undifferentiated (Fig. 4) according to Edmonson [12]. Animals which developed HCC by 8–10 months were mostly of grade III and some of grade IV. High incidence of AFP levels were observed in grade III where the values were 250–1110 μg/ml. Grade II HCC showed levels between 7 and 15 μg/ml. The synthesis of AFP was comparatively less marked in grades I and IV. Two tumors were recorded in aflatoxin B1 group: one is HCC of grade III with serum AFP levels less than 2 μg/ml and the other is a cholangiocarcinoma with no rise of AFP levels.

The findings were correlated using immunoperoxidase staining for AFP synthesis in these tumor tissues (Fig. 5). The poorly differentiated HCC showed intensive staining reaction indicating that all the cells synthesize AFP. Whereas in the tumors of grades I and II the AFP cells were distributed either singly or in clusters with intermixing AFP negative cells.

DISCUSSION

Early appearance of serum AFP in experimental rats has been designated as "Primary reaction" [13]. This is characterized by proliferation of the controversial "oval cells" and small hepatocytes in the liver [14]. The sequential changes observed in the rat liver during oncogenesis are as follows: injury, regeneration, proliferation of neoplastic nodule and ultimately to frank HCC [15]. In this study the synthesis of AFP started from first month onwards ranging 1–4 μg/ml (Fig. 2) presumably associated with the onset of regeneration. Sell *et al.* [16] observed a marked rise of AFP synthesis after partial hepatectomy in rats during the period of regeneration and serum AFP levels were six times the normal values. The observed rise in AFP during the "primary reaction" is most likely due to regeneration. In the present study 12/30 animals were fed the carcinogenic diet for 10 weeks only yet developed HCC. These observations are in accordance with those made by Hirai *et al.* [13], in which the DAB containing diet was replaced by a normal diet before the 7th week: none of the animals developed HCC; however, when DAB feed was replaced on the 12th week, HCC developed in most of the animals. It possibly indicated that the carcinogen has to act on the regenerating cells to cause malignant changes. Mice given 0.15% thioacetamide (TAA) after partial hepatectomy developed HCC earlier than those with TAA diet alone [17]. It may thus be speculated that the transitory AFP elevation in patients with liver cirrhosis or hepatitis indicate a "primary reaction". These patients may eventually develop HCC under the influence of some unknown carcinogenic factors

Table 1. *Showing the serum AFP levels, time of appearance of HCC and histological grading in various groups of animals in azodye carcinogenesis*

Group	AFP response Biphasic or monophasic	Maximum AFP levels (μg/ml)	Time of appearance of HCC (months)	Histological grading
A.	Biphasic	250–1110	8–10	Grades III and IV
B.	Biphasic	7–15	16–24	Grades I and II
C.	Monophasic	7–15	16–24	Grades I and II
D.	Monophasic	2–4	—	—
E.	None	<0.1	—	—

like naturally occurring aflatoxin or nitrosamine.

In the present study it is possible to divide the tumor-bearing animals into two distinct groups- "quick" and "slow" responders on the basis of their capacity to synthesize AFP. In "quick responders" the tumors developed by 8–10 months with high AFP values ranging from 250 to 1110 μg/ml (Group A). Majority of the tumors in this group were poorly differentiated HCC. The slow responders on the other hand developed HCC after 16 months and they were better differentiated and the serum AFP levels were significantly lower (15 μg/ml) than the other group. Relatively poorly differentiated (grade III) are able to synthesize AFP in larger-quantity. Highly anaplastic HCC behaving as quick responders, yet could not attain very high levels of AFP. (Table 1). These observations are consistent with those of Takahashi [18] who reported similar findings in human HCC. There were no tumors in groups D and E, though some of the animals did show transient initial rise of AFP.

There is an apparent discrepancy regarding AFP levels in aflatoxin induced hepatoma. Kroes and his coworkers [7] for first time demonstrated the rise of serum AFP in aflatoxin induced HCC in young Fischer strain rats and the reported levels ranged from as low as 1 μg to as high as 100 μg [19]. In our study 1/10 rats developed HCC only at the end of 26 months and the serum AFP levels did not exceed 2 μg/ml. This difference could possibly be attributed to the strain used, lower dose of aflatoxin B1, higher age of the animal in our study as well as tempo of carcinogenesis during aflatoxin B1 therapy.

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Oestrogen Receptors, Cellularity, Elastosis and Menstrual Status in Human Breast Cancer*

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Abstract—*In a study of* 128 *human primary breast cancers, a significant association has been shown between tumour cellularity and the level of oestrogen receptor activity (ORA) in tumours in which ORA was present, although cellularity was not related to the presence or absence of detectable ORA. In contrast, there was no association between the grade of elastosis and the level of ORA, but a highly significant association was observed between the grade of elastosis and the presence or absence of detectable ORA. Tumours containing large amounts of elastosis were more common in postmenopausal* (22/84) *than in premenopausal* (1/35) *women.*

INTRODUCTION

Two FEATURES of human breast cancer correlated with response to endocrine therapy, oestrogen receptors [1, 2] and elastosis [3], have themselves been shown to be associated [4]. The present report further examines elastosis and oestrogen receptor activity in relation to cellularity and menstrual status.

MATERIAL AND METHODS

One hundred and twenty-eight primary breast tumours were examined, 84 from postmenopausal patients, 35 from premenopausal and menopausal patients (patients whose last period was within 2 yr of the biopsy) and 9 from patients whose menstrual status could not be accurately classified.

Oestrogen receptor activity (ORA) was measured by saturation analysis of free and bound hormones with dextran-coated charcoal using methods previously described [5], and elastosis was demonstrated using a modified Gomori aldehyde-fuchsin stain [4]. The ORA was categorized as plus (detectable, concentration of oestrogen receptor binding sites, Po, greater than 0.1 fmole/mg wet wt of tissue) or minus (non-detectable, Po less than 0.1 fmole/mg wet wt of tissue). The tumours were graded according to the amount of elastosis into categories 0, 1 and 2, where 0 indicates that elastosis was not demonstrable, 1 indicates that elastosis was present in small or moderate amounts and 2 indicates the presence of a gross amount of elastosis. Elastosis was independently assessed blind by three observers, agreement between all three observers being 81% and between two of three observers 100%. A majority decision was accepted. Tumour cellularity was assessed using three categories: 1, 2 and 3, where 1 indicates a low, 2 a moderate and 3 a high tumour: normal tissue ratio. Cellularity was independently assessed blind by two observers, the agreement being 74%. Where disagreement occurred, the slides were re-read blind and classified by agreement.

The relationships between the presence or absence of detectable ORA and the grades of (a) cellularity and (b) elastosis were examined using chi-squared tests (see Tables 1 and 2). In addition, the relationships between the oestrogen receptor concentration in the ORA plus group and the grades of (a) cellularity and (b) elastosis, were analysed statistically. Because the receptor concentrations were not distributed symmetrically, the common median was regarded as a useful measure of the centre of distribution. The null hypothesis that the observations were derived from three distributions (the three grades of cellularity or elastosis) with a common median was examined using a chi-squared test (see Tables 3 and 4 and Figs. 1 and

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Table 1. Relationship between presence or absence of oestrogen receptors and cellularity in 128 primary breast cancers

Oestrogen* receptor category	Cellularity gradings†		
	1	2	3
Postmenopausal			
+	18	33	18
−	3	9	3
Premenopausal and menopausal			
+	2	17	2
−	3	7	4
Unclassified			
+	1	3	1
−	0	1	3
Total			
+	21	53	21
−	6	17	10

*Oestrogen receptor activity + > and − < 0.1 fmole binding sites/mg wet wt of tumour.
†Cellularity grading: 1 = low, 2 = moderate, 3 = high, tumour/normal tissue ratio.

Table 2. Relationship between presence or absence of oestrogen receptors and grade of elastosis in 128 primary breast cancers

Oestrogen* receptor category	Elastosis grading†		
	0	1	2
Postmenopausal			
+	7	42	20
−	5	8	2
Premenopausal and menopausal			
+	2	18	1
−	6	8	0
Unclassified			
+	1	3	1
−	3	1	0
Total‡			
+	10	63	22
−	14	17	2

*Oestrogen receptor activity + > and − < 0.1 fmole binding sites/mg wet wt of tumour.
†Elastosis grading: 0 = none, 1 = small or moderate amount, 2 = gross amount.
‡Association between grade of elastosis and presence of receptors significant at $P < 0.001$ by χ^2 test.

2), a statistical procedure known as the extension of the median test [6].

The results for the tumours from postmenopausal women alone as well as those for all tumours were analysed statistically, but the number of specimens in the premenopausal/menopausal groups was too small to be thus analysed.

Table 3. Relationship between concentration *of oestrogen receptors and cellularity in 95 receptor-positive primary breast cancers*

Oestrogen receptor concentration (fmole/mg tumour)	Grade of cellularity		
	1	2	3
Postmenopausal			
≦3.12*	16 (9)	16 (17)	4 (9)
>3.12	2 (9)	17 (16)	14 (9)
All menstrual groups			
≦2.5*	16 (11)	26 (27)	6 (11)
>2.5	5 (10)	27 (26)	15 (10)

Tumours have been classified to show the frequency of occurrence of receptor concentrations greater or smaller than the common median (denoted by *) within each grade of cellularity. The figures in parenthesis show the expected numbers in each cell if the level of receptor activity is independent of the grade of cellularity. Deviations from the expected distribution are significant at the $P < 0.001$ and $P < 0.01$ levels for "postmenopausal" and "all menstrual" groups respectively.

Table 4. Relationship between concentration *of oestrogen receptors and grade of elastosis in 95 receptor-positive primary breast cancers*

Oestrogen receptor concentration (fmole/mg tumour)	Grade of elastosis		
	0	1	2
Postmenopausal			
≦3.12*	2 (4)	22 (22)	12 (10)
>3.12	5 (3)	20 (20)	8 (10)
All menstrual groups			
≦2.5*	4 (5)	35 (32)	9 (11)
>2.5	6 (5)	28 (31)	13 (11)

Tumours have been classified to show the frequency of occurrence of receptor concentrations greater or smaller than the common median (denoted by*) within each grade of elastosis. The figures in parenthesis show the expected numbers in each cell if the level of receptor activity is independent of the grade of elastosis.

RESULTS

(A) *Oestrogen receptor activity and cellularity*

The results are summarized in Tables 1 and 3. No association was observed between the presence or absence of detectable ORA and the grade of cellularity for either the postmenopausal group ($\chi^2 = 0.97$) or for all the tumours examined ($\chi^2 = 0.94$). In contrast, in the ORA plus group, the level of activity was positively correlated with the grade of cellularity (see Table 3 and Fig. 1) in both the postmenopausal group ($P < 0.001$) and all the tumours examined ($P < 0.01$).

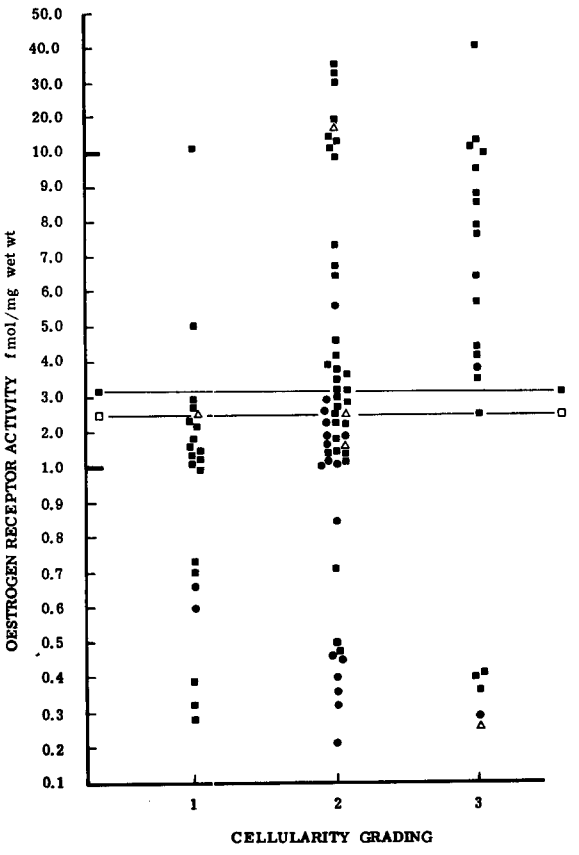

Fig. 1. Scatter plot showing the distribution of ORA for receptor-positive tumours in the three grades of cellularity. The common median P_0 value for the postmenopausal group (■———■) and all tumours (□———□) is shown. The number of tumours in each grade with a P_0 value greater or smaller than the common median is shown in Table 3. The tumours are categorized by the following symbols: (■) postmenopausal, (●) premenopausal, (△) menstrual status not determined.

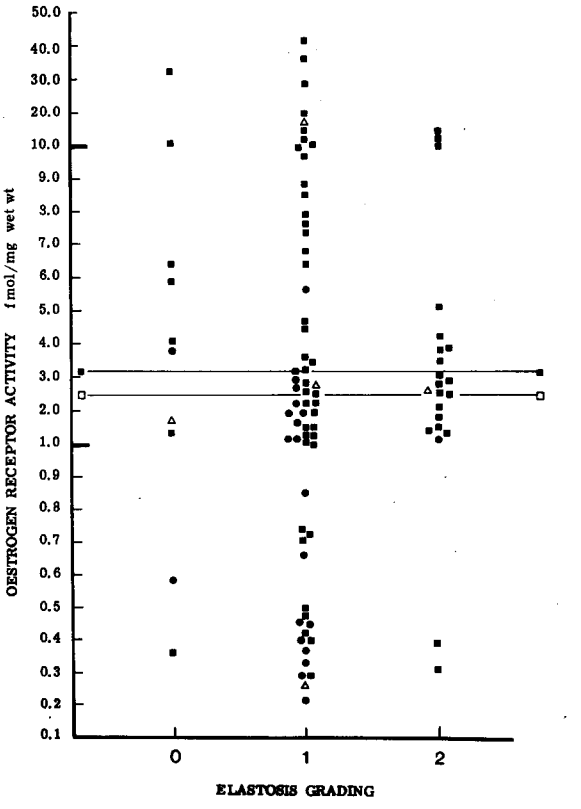

Fig. 2. Scatter plot showing the distribution of ORA for receptor-positive tumours in the three grades of elastosis. The common median P_0 value for the postmenopausal group (■———■) and all tumours (□———□) is shown. The number of tumours in each grade with a P_0 value greater or smaller than the common median is shown in Table 4. The tumours are categorized by the following symbols: (■) postmenopausal, (●) premenopausal, (△) menstrual status not determined.

(B) *Oestrogen receptor activity and elastosis*

The results are summarized in Table 2, and show a definite association between the presence or absence of detectable ORA and the grade of elastosis in all the tumours examined ($P<0.001$), although this did not attain statistical significance in the postmenopausal group alone ($P<0.1$, >0.5). In contrast, there was no association (see Table 4 and Fig. 2) between the level of activity in the ORA plus group and the grade of elastosis in either the postmenopausal group ($\chi^2=2.05$) or the total ($\chi^2=1.89$).

(C) *Elastosis and cellularity*

The results are summarised in Table 5, and there is no apparent correlation between the grades of cellularity and elastosis. According with the definition of the gradings, it was not possible to observe both high cellularity (grade 3) and gross elastosis (grade 2) in the same tumour.

Table 5. The frequency of concurrence of each grade of elastosis and cellularity in 128 human primary breast tumours

Elastosis grading	Cellularity grading		
	1	2	3
Postmenopausal			
0	1	7	4
1	12	21	17
2	8	14	0
Premenopausal and menopausal			
0	2	2	4
1	3	21	2
2	0	1	0
Unclassified			
0	0	2	2
1	0	2	2
2	1	0	0
Total			
0	3	11	10
1	15	44	21
2	9	15	0

DISCUSSION

The purpose of this study was to investigate the interrelationships between oestrogen receptor activity, menstrual status, elastosis and cellularity in human primary breast cancer. ORA was measured quantitatively and objectively, whereas elastosis and cellularity were assessed subjectively. While subjective assessments can be biased, in the present study each feature was classified into three categories and assessed blind. The only apparent source of bias was a tendency to "play safe", giving a preponderance of results in the middle category for each feature.

The results show that the grade of cellularity does not influence the classification of ORA as plus or minus, concurring with previous findings [7–9]. However, in the present study, in tumours in which ORA was detectable, a highly significant association was observed between tumour cellularity and the level of ORA, confirming an earlier observation in multiple sections from 11 large primary tumours [10]. This finding is, however, in contrast to two previous reports [7, 8], while Rosen *et al.* [11] observed a trend but did not consider it significant, agreeing in general with these two previous reports. Terenius *et al.* [9] found a statistically significant difference in the level of ORA between tumours of high and moderate cellularity, but not between tumours of high and low cellularity (possibly because of the small numbers in the low cellularity group). Thus, the present study shows that although cellularity does not affect the rate for detection of receptor-positive tumours, it does influence the level of ORA when present. The findings are important if patients are to be selected for endocrine therapy on the basis of the concentration of oestrogen receptors, rather than the presence or absence of detectable ORA. The importance of relating clinical response to receptor concentration rather than presence or absence of detectable receptor has already been demonstrated [12, 13].

In contrast to cellularity, a significant association was shown between the grade of elastosis and the presence or absence of detectable ORA, but not with the level of ORA. Since the majority of this group of tumours has been described in our previous report [4], this observation simply extends our previous findings. One new finding was made on the relationship between elastosis and menstrual status. It was observed (Table 2) that 22/84 (26%) of the postmenopausal, in contrast to 1/35 (3%) of the premenopausal/menopausal group, contained a gross amount (grade 2) of elastosis. This finding provides further indirect evidence that there is a relationship between elastosis and the hormonal environment in human breast tumours. No satisfactory explanation can be given at present for these findings.

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The Effect of Bleomycin and its Combined Effect with Radiation on Cultured Chinese Hamster Cells V-79

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Abstract—*The effect of bleomycin and combined effect of it with radiation on Chinese hamster cells V-79-BlM was studied.*

The survival curve of cells exposed to bleomycin showed slightly upwards-concaved firstly, then followed by exponential portion from a surviving fraction of 0.2, *and* D_0 *of terminal slope is* 185 μg/ml. *The result of two fractional exposure of bleomycin in the interval of* 1 *or* 5 hr *demonstrated less cell survivals than in single exposure. In combined treatment of bleomycin with radiation the decrease of* D_0 *was recognized, however* D_0 *was not changed compared with X-ray survival curve.*

INTRODUCTION

BLEOMYCIN is an anti-tumour drug isolated by Umezawa [1, 2] from cultures of *Streptomyces verticillus*. This antibiotic, a complex glycopeptide, has strong anti-tumour activities to human squamous carcinoma. Bleehen *et al.* demonstrated the interaction on *E. coli* B/r, however, they failed to show interactions on four of mammalian cells tested [3]. Radiation-drug interaction is of considerable interest because of a clear interaction between drug and radiation may show the way to enhance the possibility of curing certain cancers. Elkind and Sakamoto [4] suggested that interaction between radiation and drugs was unlikely when drug gave an exponential dose–response curve [4, 5] e.g. the survival curve of Chinese hamster cells treated with nitrogen mustard is exponential and this system does not show the drug-radiation interaction, but the survival curve of Chinese hamster cells exposed to actinomycin D is the threshold type, actinomycin D has shown strong interaction with radiation on the cells.

To study the relation between the mode of survival curve and the interaction is of interest. In the present paper, the effect of another drug, namely bleomycin, and its combined effect with 200 kVp X-ray on cultured Chinese hamster cells V-79 will be shown.

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MATERIAL AND METHODS

The experimental system used in this experiment is essentially the same as described by Elkind *et al.* [6, 7] therefore it will only be presented briefly.

Cells and their cultivation

A number of subclones of Chinese hamster cells designated originally V-79-1 by Elkind has been used. The doubling time of these cells when grown on glass dishes is 11–12 hr. They have a fibroblast-like morphology and produce colonies of about 2 or 3 mm dia in 9–10 days. Colony-forming ability was used as a measure of the cell's survival *in vitro*. Using a modified Eagle's medium [8] to which 15% fetal calf serum was added (EM-15), cells plated in appropriate numbers in 6 cm Petri dishes were grown overnight in a CO_2 incubator at 37°C before an experiment was started. This procedure was used to help insure that the distribution of cells throughout their growth cycle (i.e. the age density distribution) was close to that resulting from asynchronous, log-phase growth and hence fairly reproducible from experiment to experiment. During this overnight growth period, initially single cells formed micro-colonies, on average about three cells; consequently the experiments started with populations of micro-colonies rather than single cells.

Following a given treatment sequence, cells were incubated long enough for a maximum yield of colonies. They were then stained with

methylene blue and counted. Results are expressed as the fraction of cells surviving a given treatment relative to the number in the initial suspension plated, which were able to form colonies. (The percentage of colony formers in the starting suspension is designated the plating efficiency, P.E.)

Bleomycin treatment

The stock solution of bleomycin was prepared at a concentration of 5.0 mg/ml in saline and kept frozen between experiments. For use, stock solution was diluted in Eagle's medium with 15% fetal calf serum to 10 times higher than the final concentrations required. They were kept on ice and bleomycin treatment was made by adding 0.5 ml aliquots of appropriate drug solution to 4.5 ml of medium at 37°C for 3 hr. The reason why 3 hr was adapted as a treating time is that three hours was the minimum time to bring the survival to an exponential portion in the dose–response curve (Fig. 1). At the end of the treatment, bleomycin containing medium was sucked off, the cells were rinsed once with buffer containing 5% EM-15 and supplied with fresh medium at 37°C and incubated to observe eventual colony formation.

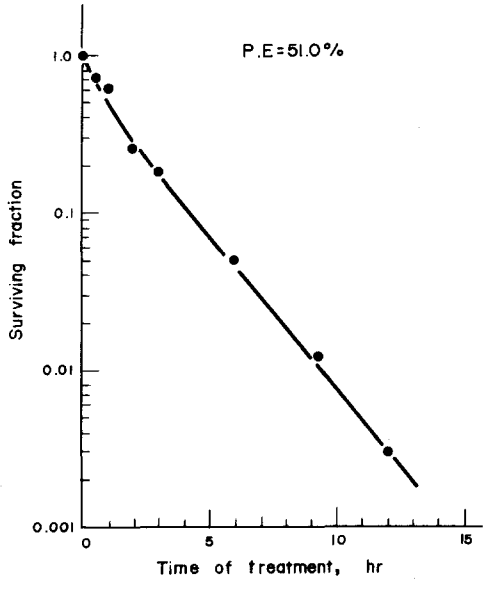

Fig. 1. Survivals of Chinese hamster cells treated with bleomycin in various treating time. The surviving fraction was the ratio of plating efficiency of a treated cell over that of the non-treated control. The survival curves were always constructed by plotting survival against total dose of the drug used. (Standard errors are shown where the means of several points are plotted.)

X-irradiation

The cells in Petri dishes with EM-15 medium were irradiated at 37°C. X-rays were generated by a therapy machine operated at 200 kVp and 20 mA and were filtrated through 0.5 mm Cu and 1.0 ml Al. The exposure dose rate, measured with a Victoreen Radocon II Integrating Ratemeter (Model 555), was 60 rad/min; the FSD was 50 cm. Immediately after exposure at room temperature, medium was sucked off and fresh medium kept at 37°C was applied to the dish for final incubation.

In the case of combined treatment of X-ray with bleomycin, if the first treatment was X-irradiation, medium was removed immediately after irradiation, fresh medium was put into the dish and bleomycin was added, 3 hr later bleomycin containing medium was taken away, cells were rinsed once with buffer and supplied with fresh medium and incubated for colony formation.

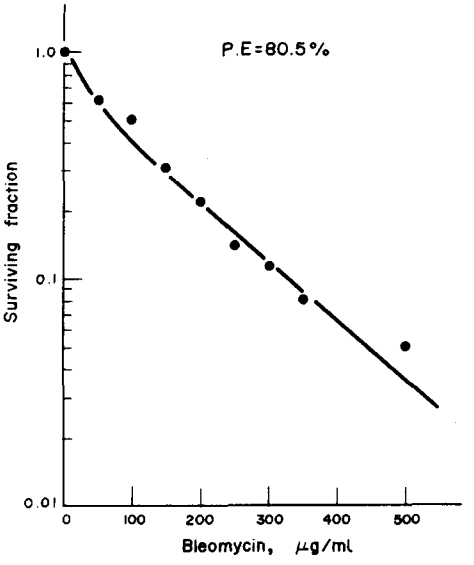

Fig. 2. Dose–survival curve of Chinese hamster cells treated with bleomycin for 3 hr.

In the experiment that bleomycin was used as the first treatment, medium was sucked off from dish containing bleomycin after 3 hr treatment, cells were rinsed once, then fresh medium was refed. The medium was replaced again with fresh medium for final incubation after X-ray exposure.

RESULTS

Survivals after bleomycin treatment

In Fig. 1, the survival of Chinese hamster cells V-79-BIM treated with bleomycin of 200 μg/ml is plotted against the time of treatment (time-inactivation curve). The curve begins with a slightly upward-concave curve, followed with an exponential declining line from a surviving fraction of 0.2. Figure 2 demonstrates

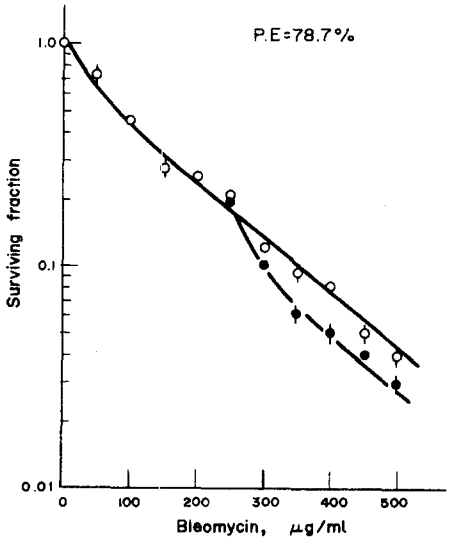

Fig. 3. Survival curve of Chinese hamster cells V-79-BlM after single and double treatment with bleomycin.
○: *Survival of cells treated with the drug at various concentrations for* 3 hr.
●: *Survivals of the cells which were treated first with the drug at* 250 μg/ml *for* 1.5 hr *and followed by a second treatment for* 1.5 hr *with the drug of various concentrations* 1 hr *after the first treatment.*

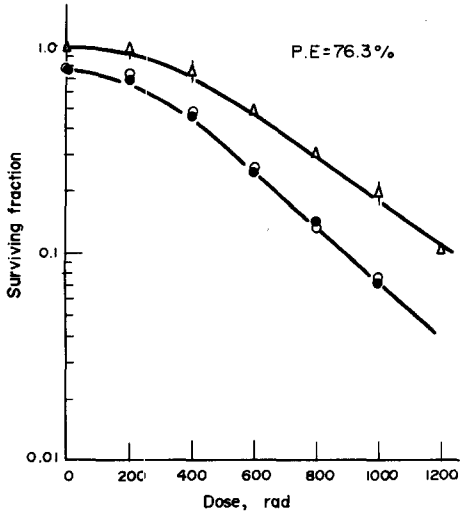

Fig. 4. Survival of Chinese hamster cells V-79-BlM after combined treatment of bleomycin and X-ray.
△: *Survivals of cells exposed to* 200 kVp *X-rays of various doses.*
○: *Survivals of cells exposed to various doses of X-rays immediately after treatment with bleomycin of* 100 μg/ml *for* 3 hr.
●: *Survivals of cells treated with bleomycin of* 100 μg/ml *for* 3 hr *immediately after X-ray exposure of various doses.*

the survival curve of cells to single dose of bleomycin for a fixed treatment time of 3 hr. The curve is similar to the time–inactivation curve of Fig. 1. The D_0 of the exponential portion of Fig. 2 is 185 μg/ml. Since these survival curves are quite different from a sigmoid type of a survival of X-irradiated Chinese hamster cells, it is difficult to assume the existence of Elkind-type recovery (an increased survival from recovery of X-ray induced sublethal type of damages). In order to confirm whether or not Elkind-type recovery is in bleomycin induced damage, split dose experiments were performed. In Fig. 3, the open circles are the survivals of cells treated with single doses of bleomycin for 3 hr and the closed circles are the survivals of cells, which were firstly treated with 250 μg/ml of bleomycin for 1.5 hr, rinsed and maintained in drug-free medium for 3 hr, and exposed second time with graded doses of bleomycin for 1.5 hr. The closed circle at 250 μg/ml bleomycin in Fig. 3 is that received the first 250 μg/ml bleomycin for 3 hr, followed by 3 hr of the non-treatment and by 1.5 hr of another non-treatment (0 μg/ml bleomycin). The results of split dose experiments are opposite to what

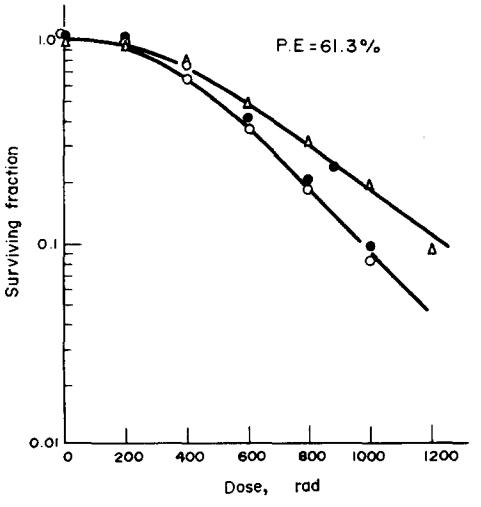

Fig. 5. Survival of Chinese hamster cells V-79-BlM after combined treatment of bleomycin and X-ray.
▲: *Survivals of cells exposed with X-rays of various doses.*
○: *Survivals of cells exposed to various doses of X-rays immediately after treatment with bleomycin of* 30 μg/ml *for* 3 hr.
●: *Survivals of cells treated with bleomycin of* 30 μg/ml *for* 3 hr *immediately after X-ray exposure of various doses.*

one sees in split dose experiments with X-rays. The reasons why the cell survival rate is less in two-fractionated than in single exposure of bleomycin are not clear. The residual damage in cells by first treatment may relate to the low survival ratio in the fractionated treatment.

Combined treatment

In Fig. 4, open triangles indicate the survivals of cells irradiated with 200 kVp X-rays, closed circles are those of the cells irradiated with X-rays first and treated with bleomycin of 100 μg/ml for 3 hr after X-irradiation, and open circles are those of the cells treated with 100 μg/ml bleomycin for 3 hr and followed with graded doses of X-rays.

Figure 5 is another set of experiments, where 30 μg/ml of bleomycin were used instead of

100 μg/ml. D_0s of X-rays survival curves in Figs. 4 and 5 are 390 and 370 rad respectively, these values are almost three times as large compared with the value obtained on the same cell line by Elkind and Sakamoto [4]. However, D_0 values in present paper are reproducible, Chinese hamster cells which are cultivated in my laboratory may become radio-resistant compared to the original cells.

In Figs. 4 and 5, bleomycin treatments before X-irradiation were not significantly distinguishable from the effects of bleomycin treatment after X-irradiation. The effect of the post- and pre-X-irradiation treatment of bleomycin was apparent in the D_0 values of the survival curves in Fig. 4, D_0 of 390 rad for X-irradiation alone was decreased to 308 rad with bleomycin treatment, while in Fig. 5, D_0 of 370 rad for X-rays alone was decreased to 270 rad with bleomycin treated group. However, bleomycin treatments affected little on the D_q values of X-irradiated cells.

DISCUSSION

The survival curves of Chinese hamster V-79 cells are made up of an initial rapid decline (upward-concaved), followed by an exponential decline. This type of survival curves is observed not only in the relation to a fixed bleomycin with various treatment times (Fig. 1), but also in the relation to various bleomycin concentration with a fixed treatment time (Fig. 2). Two curves indicated a shift to an exponential decline at around survival fractions of 0.2–0.3. Previously, Terasima *et al.* [9] obtained this type of survival curve in mouse L cells from varied treatment times, Sakamoto in a murine squamous cell carcinoma *in vivo* [10], and Barranco *et al.* in CHO cells *in vitro* [11].

It is noted that no such survival curves were found in other drugs with Chinese hamster cells (e.g. actinomycin D [3], nitrogen [4] mustard, neocarzinostatin [12] and daunomycin [13].

Since this type of survival curve seemed to be quite opposite to what one sees in the survival curve of X-rays (e.g. Figs. 4 and 5), where there is a shoulder, followed by a rapid exponential decline in their survival curves. Figure 3 shows that introduction of a time interval between two doses did produce an initial rapid decline portion. This seems to deny the existence of "sublethal damage (recoverable) of Elkind type" with bleomycin. Its outcome is a suggestion that bleomycin treatment of cancer patients (if cancer cells has similar survival curves) should be made the most effectively if treating the cells with small doses (say, sufficient to bring them to the exponential portion of the survival curve) in intervals of 3–6 hr.

The next question is what would be the bleomycin resistant component in the survival curve? Terasima *et al.*, noting the similar type of the survival curve in L cells, suggested several possibilities: a population made up of bleomycin sensitive and resistant cells, an existence of a saturation dose level, over which bleomycin affects cells ineffectively, and an induction of bleomycin-resistancy in the treated cell population. Humphrey *et al.* found that bleomycin treatment inducement blockage of cell progress at S and G_2 stages [11]. However, our results are not sufficient to support any hypothesis at this stage.

Since survival curve with bleomycin was different from actinomycin D, nitrogen mustard and neocarzinostatin and daunomycin, the combined effects with X-ray were carried out. In the previous works of Matsuzawa *et al.*, bleomycin had potentiating effect on X-rays especially in the post-irradiation treatment. While Terasima found little potentiating effects in mouse L cells, Bleehen *et al.*, reported no potentiating effects in HeLa cells, and *E. coli.* Sakamoto *et al.* [12] were unable to demonstrate a potentiating effect on a murine squamous cells carcinoma *in vivo*. In the present experiments, two concentrations bleomycin were used. One is a very low concentration of 30 μg/ml, which hardly kills the cells and the other is 100 μg/ml, which killed most of bleomycin sensitive cells, leaving bleomycin-resistant cells. Under these conditions, post- and pre-irradiation treatments with bleomycin showed no effects on D_q values of X-irradiated cells, and some 20–29% decrease in the D_0 portions of the X-irradiated cells. In other words, no effects of bleomycin on D_q seemed to suggest that bleomycin did not interfere "sublethal type of X-ray damages" and that it potentiates lethal type of X-ray damages. Or X-ray sensitivity of two bleomycin treated populations (one with bleomycin sensitivity and the other with decreased sensitivity and mostly resistant population) was similar. It is however, noted that the extent of bleomycin potentiation was only 20–29% in reduction of D_0 values.

Acknowledgements—I am grateful to Mrs. Yukiko Matsunaga and Miss Mariko Inoue for their technical assistance and to Nippon Kayaku Co. Ltd. for supplying bleomycin.

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Letter to the Editor

Fibrinolysis in Lung Cancer

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IT IS a feature of many malignant cells that they produce elevated levels of a urokinase-like plasminogen activator which converts serum plasminogen to plasmin *in vitro* [1, 2]. Since a prominent physiological function of plasmin is the cleavage of fibrin into fibrin degradation products (FDP), elevated FDP levels might be expected to occur in association with malignancy. In fact, there is considerable evidence of elevated fibrinolytic activity in some types of cancer, as well as in human tumour cell lines in culture [3]. For example, the majority of breast carcinomas and a substantial proportion of gastrointestinal adenocarcinomas were found to have plasminogen activator properties when tested *in vitro* [4]. However, this may not be a feature of all types of cancer. Thus, in acute leukaemia there was wide variation in fibrinolytic status [5], while sarcomas have been reported to exhibit only spontaneous fibrinolytic activity unassociated with plasminogen activation [4]. The levels of FDP in serum and urine are also substantially elevated in a variety of non-cancerous conditions, including disseminated intravascular coagulation [6], pulmonary embolism [7], deep vein thrombosis and myocardial infarction [8]. Since the urine contains urokinase, and the prostate exhibits direct fibrinolytic activity [9, 10], kidney or urinary tract disease may cause elevation of urinary, but not serum FDP [11, 12]. Consequently elevated FDP levels might be predicted in patients with some types of cancer, the relation between the two being dependent upon the elimination of other potential causes.

FDP assay systems are already being used in clinical cancer studies, for example, to evaluate the success of urokinase injection following tumour resection [13]. We have investigated the potential use of FDP assay as a diagnostic aid prior to confirmation of malignant disease. Our initial investigation has been carried out on patients with suspected bronchial carcinoma, referred for bronchoscopy. Samples of blood and urine were obtained from resting patients admitted to hospital a few hours previously. The samples were immediately mixed with soybean trypsin inhibitor to prevent further plasmin-dependent fibrinolysis; it may not, however, completely inhibit fibrinolysis by other enzymes [10]. Serum was then prepared, and stored at −20°C for up to a week before assay. The results are given in Table 1 and show that 78% of patients with subsequently confirmed bronchial carcinoma had normal FDP levels. Of the remaining 22% with bronchial carcinoma who showed elevated levels of FDP in urine and/or serum, only one individual was apparently free from concurrent nonmalignant disease likely to cause elevated FDP.

We conclude that elevated FDP levels are not a feature of primary carcinoma of the bronchus in newly referred patients and that the assay has no value as a diagnostic aid in such cases. However, in view of a report of 5 epidermoid carcinomas of the lung without associated fibrinolytic properties *in vitro* [4], together with the negative findings in our series, it seems that bronchial carcinomas may not be a potent source of plasminogen activator and may differ in this respect from some other forms of cancer. An alternative possibility is that tumour-associated fibrinolytic activity is present, but restricted to the tumour site in our patients; becoming generalized only at a later stage in the disease process. This alternative possibility is supported by preliminary observations, carried out in collaboration with Mr. John D. Griffiths and Mr. Harvey White, Royal Marsden Hospital, London, on patients with colorectal carcinomas. Tumours examined so far in this series have shown high plasminogen activator activity in fibrin–agar plate assays despite the fact that neither peripheral blood nor blood drained from the venous circulation of the tumour during operation contained elevated FDP. Thus, tumour-associated elevation of vascular FDP in cancer patients may be more characteristic of relatively gross and

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generalised pathological changes than of localised fibrinolytic activity at the tumour site. This former type of change has been described by Brassinne *et al.*, who have suggested that the hypofibrinogenaemia associated with prostatic adenocarcinoma may be due to disseminated intravascular coagulation [14], possibly associated with disseminated fibrinolysis.

Table 1. *FDP Levels* of* 31 *bronchoscopy patients*

No. of patients	Lung abnormality	Urine FDP (μg/ml)	Serum FDP (μg/ml)	Other possible causes of elevated FDP
20	bronchial carcinoma	normal	—	—
2	bronchial carcinoma	normal	normal	—
1	bronchial carcinoma	20	normal	none found
1	bronchial carcinoma	—	10	trauma
1	bronchial carcinoma	2.5	—	phlebitis
1	bronchial carcinoma	10	—	renal calculi
1	bronchial carcinoma	2.5	—	cardiac failure
1	bronchial carcinoma	1.25	—	cardiomegaly, dyspnoea
2	nonmalignant condition	normal	—	—
1	nonmalignant condition	normal	normal	—

*FDP levels were measured by haemagglutination—inhibition assay using the Wellcome FDP kit HA14. Normal levels were taken to be 5 ± 3 μg/ml (serum) and 1.25 μg/ml (urine). The normal urine levels of 0.25 μg/ml exceeds test sensitivity.

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Announcement

6th MEETING INTERNATIONAL RESEARCH GROUP FOR CARCINO-EMBRYONIC PROTEINS (I.R.G.C.P.)

17–21 *September* 1978, *Marburg/Lahn, West-Germany*

Topics: Carcino-embryonic proteins: 1. Clinical application; 2. Biology, immunochemistry and significance in embryo- and carcinogenesis; 3. Methods, standardization.

Information: Prof. Dr. F.-G. Lehmann, Dept. of Medicine, E. Mannkopffstraße 1, D-3550 Marburg/Lahn, West-Germany.

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Recent Journal Contents (1978)

International Journal of Cancer *February, 1978*

Human Cancer

J. Bara, A. Paul-Gardais, F. Loisillier and P. Burtin: Isolation of a sulfated glycopeptidic antigen from human gastric tumors. Its localization in normal and cancerous gastrointestinal tissues.

F. A. G. Teulings, H. A. Peters, W. C. J. Hop, W. Fokkens, W. G. Haije, H. Portengen and B. van der Werf-Messing: Urinary excretion of tryptophan metabolites in patients with cancer of the bladder.

D. K. Hossfeld and C. G. Schmidt: Chromosome findings in effusions from patients with Hodgkin's disease.

L. Einhorn and I. Ernberg: Induction of EBNA precedes the first cellular S-phase after EBV-infection of human lymphocytes.

H. Pfister and H. zur Hausen: Seroepidemiological studies of human papilloma virus (HPV-1) infections.

S. Morikawa, E. Tatsumi, M. Baba, T. Harada and K. Yasuhira: Two E rosette forming lymphoid cell lines.

J. D. Rowley and A. de la Chapelle (Eds.): Chromosomes in leukemia. General Report of the Frist International Workshop.

Experimental Cancer

A. Colombatti, A. De Rossi, B. A. Taylor, L. Chieco-Bianchi and H. Meier: Relationship between Moloney MSV tumor resistance and endogenous virogene expression in AKR mouse strain and its hybrids.

B. A. Laishes, E. Roberts and E. Farber: *In vitro* measurement of carcinogen-resistant liver cells during hepatocarcinogenesis.

M. Kende, B. Sass, S. Hino, R. M. Donahoe and G. J. Kelloff: Type-C virus structural antigens on the surface of the infected cell as determined by a humoral cytotoxicity assay.

J. R. Oehler, L. R. Lindsay, M. E. Nunn and R. B. Herberman: Natural cell-mediated cytotoxicity in rats. I. Tissue and strain distribution, and demonstration of a membrane receptor for the Fc portion of IgG.

J. R. Oehler, L. R. Lindsay, M. E. Nunn, H. T. Holden and R. B. Herberman: Natural cell-mediated cytotoxicity in rats. II. *In vivo* augmentation of NK-cell activity.

J. R. Oehler and R. B. Herberman: Natural cell-mediated cytotoxicity in rats. III. Effects of immunopharmacologic treatments on natural reactivity and on reactivity augmented by polyinosinic-polycytidylic acid.

D. L. Putman, P. D. Kind, A. Goldin and M. Kende: Adoptive immunochemotherpay of a transplantable AKR leukemia (K36).

G. Y. Gillespie, F. C. Jensen and S. W. Russell: B-tropic oncornavirus production by BALB/c methylcholanthrene-induced sarcoma cells.

J. A. Jones, G. Robinson, R. C. Rees and R. W. Baldwin: Immune response of the draining and distal lymph nodes during the progressive growth of a chemically-induced transplantable rat hepatoma.

G. Scalabrino, H. Pösö, E. Hölttä, P. Hannonen, A. Kallio and J. Jänne: Synthesis and accumulation of polyamines in rat liver during chemical carcinogenesis.

M. J. Rogers, L. W. Law, M. Prat, S. Oroszlan and E. Appella: Separation of the tumor rejection antigen (TSTA) from the major viral structural proteins associated with the membrane of an R-MuLV induced leukemia.

J. D. Shaposhnikov and E. A. Ratovitski: Polyadenylated RNA molecules and polyribosomes in tumours of chemical and viral origin.

British Journal of Cancer *March*, 1978

J. Hilgers, P. J. van der Sluis, W. J. van Blitterswijk and P. Emmelot: Membrane fluidity, capping of cell surface antigens and immune response studied on leukaemia (GRSL) cells.

R. E. Nyholm and G. A. Currie: Monocytes and macrophages in malignant melanoma. II. Lysis of antibody-coated human erythrocytes as an assay of monocyte function.

M. F. A. Woodruff, V. Whitehead and G. Speedy: Studies with a spontaneous mouse tumour. I. Growth in normal mice and response to *Corynebacterium parvum*.

H. C. Ho, Mun H. Ng and K. C. Kwan: Possible factors affecting serum IgA antibody responses against Epstein–Barr viral capsid (VCA) antigens in nasopharyngeal carcinoma (NPC) patients.

L. Weiss and D. Glaves: Alterations in the arrest patterns of circulating lymphosarcoma cells in tumour-bearing mice produced by previously injected cell suspensions.

B. Maat: Extrapulmonary colony formation after intravenous injection of tumour cells into heparin treated animals.

O. I. Epifanova, I. N. Smolenskaya and V. A. Polunovsky: Responses of proliferating and non-proliferating Chinese hamster cells to cytotoxic agents.

A. G. Levis, V. Bianchi, G. Tamino and B. Pegoraro: Cytotoxic effects of hexavalent and trivalent chromium on mammalian cells *in vitro*.

J. V. Watson, P. Workman and S. H. Chambers: Differences in esterase activity between exponential and plateau growth phases of EMT6/M/CC cells monitored by flow cytofluorimetry.

R. A. Bender and D. M. Makula: The methotrexate–dihydrofolate reductase interaction and its effect on DNA and tetrahydrofolate synthesis in L1210 cells *in vitro*.

B. K. Jones and D. E. Hathway: Species differences in the metabolism of vinylidene chloride between mice and rats.

W. H. Butler: Long term effects of phenobarbitone-NA on male Fischer rats.

T. A. Hince and J. P. Roscoe: Fibrinolytic activity of cultured cells derived during ethylnitrosourea-induced rat brain carcinogenesis.

C. Williams and S. K. Carter: Management of trials in the development of anti-cancer chemotherapy.

G. W. Kneale and A. M. Stewart: Pre-cancers and liability to other diseases.

Short Communications

L. Cercek, B. Cercek, G. Bazill, J. P. Dickinson, G. Read and L. G. Lajtha: Tumour specificity of cancer basic proteins (CaBPs) as detected by the SCM test.

F. Emmrich, J. Irmscher, M. Kotzsch, M. Muller, and H. Ambrosius: Leucocyte migration inhibition under agarose versus MEM test for detection of tumour-associated antigens.

M. Das and S. E. Livingstone: Metal chelates as anti-cancer agents. Part I. Cytotoxic action of some transition metal chelates of Schiff bases derived from *S*-methyldithio-carbazene.

Failure to transfer immunity to a syngeneic rat sarcoma from mother to young by suckling.

Book Reviews

B. Lord: Cell separation methods—Workshop.

O. G. Dodge: Histological typing of testis tumours—F. K. Mostofi.

R. Harris: HLA and malignancy—G. P. Murphy, E. Cohen, J. E. Fitzpatrick and D. Pressman.

G. M. Taylor: Conditions for T cell activation.

H. Bush: Chemotherapy of cancer—S. K. Carter, M. T. Bakowski and K. Hellman.

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Papers to be Published

L. Olsson, P. Ebbesen, N. Kiger, I. Florentin and G. Mathé

The antileukemic effect of systemic non-specific BCG-immunostimulation vs systemic specific immunostimulation with irradiated isogeneic leukemic cells.

G. Rosset, J.-J. Farquet and A. Cruchaud

The serum immunoglobulins in lymphoproliferative malignancies.

J. S. Tobias, P. F. M. Wrigley and F. O'Grady

Bacterial infection and acute myeloblastic leukaemia: an analysis of two hundred patients undergoing intensive remission induction therapy.

S. Pavel, B. Matouš, J. Duchoň and Z. Schippelová

Melanogenuria in hamsters with transplantable melanoma.

A. Tagliabue, G. Allesandri, N. Polentarutti, A. Mantovani, E. Falantano, A. Vecchi, S. Garattini and F. Spreafico

The immunostimulatory activity of 3-(*p*-chlorophenyl)-2,3-dihydro-3-hydroxythiazole [3,2-α]-benzimidazole-2-acetic acid (NSC 208828).

M. Bistrović, B. Nagy, Ž. Maričić and K. Kolarić

Interaction of adriamycin and radiation in combined treatment on mouse L-cells.

G. V. Sherbet and M. S. Lakshmi

Malignancy and prognosis evaluated by an embryonic system. Grading of breast tumours.

N. Deshpande, I. Mitchell and D. Hughes

Effect of tamoxifen pre-treatment on the retention of tritiated oestradiol and 5α-dihydrotestosterone and on glucose metabolism in human breast carcinomas.

N. Deshpande, I. Mitchell, R. Millis and S. Kumaoka

Glucose metabolism in British and Japanese breast carcinomas.

J. Mattern, K. Wayss and M. Volm

Improvement of therapeutic effects of cytosine-arabinoside on Walker-carcinosarcoma after different synchronization with hydroxyurea.

K. Breur, P. Cohen, O. Schweisguth and A. M. M. Hart

Irradiation of the lungs as an adjuvant therapy in the treatment of osteosarcoma of the limbs. An E.O.R.T.C. Randomized study.

M. Dosogne-Guérin, A. Stolarczyk and A. Borkowski

Prospective study of the α and β subunits of human chorionic gonadotrophin in the blood of patients with various benign and malignant conditions.

G. Stathopoulos, H. E. M. Kay, I. M. E. Hamlin and A. M. McKay

Surface characteristics of lymphoma cells in relation to treatment, response and survival.

M. H. N. Tattersall, M. Jarman, E. S. Newlands, L. Holyhead, R. A. V. Milstead and A. Weinberg

Pharmaco-kinetics of melphalan following oral or intravenous administration in patients with malignant disease.

M. H. Claësson and G. R. Johnson

The effect of syngeneic lymphoid tumors upon mouse B-lymphocyte and granulocyte–macrophage colony forming cells.

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Role of Particle Size in the Formation of Respiratory Tract Tumors Induced by Benzo(a)pyrene*

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Abstract —*The role of particle size in experimental respiratory tumor formation was studied by intratracheal instillation of two different particle sizes of benzo(a)pyrene into Syrian golden hamsters. One group of hamsters received large particles* (64% *above* 10 μm), *the second group was treated with smaller-sized particles* (98% *less than* 10 μm). *Otherwise, treatment was similar. Only* 5 *respiratory tumor-bearing animals of* 48 *were found in the group receiving small particles, whereas* 31 *respiratory tumor-bearing animals of* 48 *were found in the group which received the larger particles. Analysis of the retention rate of instilled B(a)P showed that small particles cleared much more rapidly from the lungs than did the larger particles.*

INTRODUCTION

ADDITION of a carrier dust to a suspension of a polycyclic hydrocarbon in the experimental induction of respiratory tumors by intratracheal (i.t.) instillation using Saffiotti's methods [1] repeatedly was shown to significantly affect neoplastic progression [2]. For example, only large doses of benzo(a)pyrene [B(a)P] were effective without carrier dust and the tumor incidence was low [3], while when instilled as fine particles, B(a)P failed to induce any tumors [4]. Weekly instillation of much larger B(a)P particles in small doses induced mainly tracheal tumors [5]. The penetration and retention of carcinogen in lung tissue have been associated closely with subsequent tumor formation [6, 7]. The retention time of the carcinogen was related to the total dose instilled [3], as well as to the mode of particle preparation [8].

The present study describes the method of respiratory tumor induction and the tumor types found, in relation to retention rates, when B(a)P was given in different particle sizes and evaluates the role of particle size in experimental tumor induction.

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MATERIAL AND METHODS

Animals and chemicals

Randomly bred, 6–7-week-old male and female Syrian golden hamsters from the Eppley Colony were used. They were housed by sex in groups of 6 in plastic cages on sterilized San-i-cel bedding and given Wayne diet (Allied Mills, Chicago, IL) and tap water *ad libitum*. B(a)P was obtained from Aldrich Chemical Company, Milwaukee, WI (batch no. 111237) and found 99.4% pure by thin layer chromatography. The saline solution, consisting of 0.9% NaCl, sterile and nonpyrogenic, was obtained from Baxter Laboratories, Inc. (Morton Grove, IL) and brevital sodium [sodium-α-1-methyl-5-allyl-5-(1-methyl-2-pentynyl)barbiturate] from Eli Lilly & Co. (Indianapolis, IN).

Preparation of B(a)P suspensions

Two suspensions of B(a)P in saline were prepared to obtain different particle size ranges. For group 1, B(a)P was ground in a mullite grinder 8 hr, suspended in saline and ballmilled 17 hr. For group 2, B(a)P was ground for 24 hr, suspended in saline and ballmilled 7 days. The containers, stainless steel balls and covers, were steam-autoclaved and the suspensions stored at 4°C for 2–3 weeks. Prior to each treatment they were vibrated ultrasonically for 3–4 min.

Particle sizes were determined by Coulter

Table 1. *Particle size distribution (by weight) of benzo(a)pyrene suspensions*

Group	Size (% below)					
	40 μm	30 μm	20 μm	10 μm	5 μm	1 μm
1	100	98	90	36	10	0
2	100	100	100	98	79	5

Electronics, Inc. (Hialeah, FL.). The samples were dispersed with 3–5 min of ultrasonication and run on a Coulter Counter Model TA using a biaperture technique. They pertained to individual particles rather than agglomerates. The particle size distribution for the two B(a)P suspensions is shown in Table 1.

Experimental design and conduct

The 144 hamsters were divided into 3 groups and received i.t. instillation, as described by Saffiotti *et al.* [1]. Group I received 3 mg B(a)P (large particles suspended in 0.2 ml saline) once weekly for 18 weeks. Group 2 was administered 3 mg B(a)P (small particles) suspended in 0.2 ml saline. As the solvent control, group 3 received 0.2 ml saline once weekly for 18 weeks.

The animals were weighed once weekly and checked twice daily, at which time gross observations were recorded. The animals were allowed to die spontaneously or were killed when moribund. For morphological studies at autopsy, tracheas were ligated and lungs removed *en bloc*, still fully expanded by air, and then fixed by immersion in 10% neutral buffered formalin. Histologic sections were prepared from each lobe of the lungs, larynx, trachea, stem bronchi and other organs showing gross pathologic changes. Sections were stained with hematoxylin and eosin. Other stains were used when needed.

Lung retention of B(a)P

To study the effect of different particle sizes of B(a)P on the retention rate, hamsters that received a single B(a)P instillation were serially sacrificed. The same suspension used for tumor induction was also employed in the retention rate study. For the 0 hr time determination, 3 hamsters were killed with sodium brevital within 30 sec after instillation and the lungs were placed in methylene chloride. Hamsters were also sacrificed in groups of 3 at intervals of 6 and 18 hr and 3 days. The respiratory tract, including the larynx, trachea and all lung lobes, was removed *en bloc* and placed in a "Virtis" homogenizer with 20 ml methylene chloride and homogenized for 5 min at full speed. The homogenate was then centrifuged for 15 min at 300 rev/min in a Fisher Scientific Centrifuge. The pellet which formed at the top was separated from the supernatant liquid and washed. The washings were combined with the supernatant, dried with magnesium sulfate and filtered, after which the volume was recorded. After appropriate dilution, the ultraviolet spectrum was determined and absorption at 387 nm used to calculate the B(a)P concentration. Further extraction of the pellet with methylene chloride produced no additional product. Recovery of B(a)P from the hamster respiratory tract at 0 hr consistently produced >95% of the instilled dose. Subsequent values are expressed as percent of the 0-hr recovery.

RESULTS

The survival rates of hamsters are given in Table 2. Those treated with the small particle size of B(a)P (group 2) had a median lifespan of 55 weeks, compared to 36 weeks in hamsters treated with the large particle size (group 1). The most common cause of death in group 2 was nephropathy, unrelated to treatment. Occasionally, pneumonia was seen in the solvent-treated animals. Suffocation due to neoplastic involvement of the respiratory system was occasionally encountered in group 1 and neoplastic alterations were found in many animals (Table 3).

Intratracheal instillation of large B(a)P particles induced laryngeal tumors, consisting of squamous cell papillomas in 5 animals. The trachea was the most common location for tumors and there were 12 papillomas, which were villous structures covered by squamous epithelium. There were also 20 squamous cell carcinomas, mostly well-differentiated with large keratin sheaths and 2 undifferentiated sarcomatoid tumors. Bronchial epithelial alterations, desquamation, squamous metaplasia and hyperplasia were seldom seen. The number of lung tumors was lower compared to tracheal tumors and consisted of 9 squamous cell carcinomas, similar to those of the trachea, 2 papillomas, 2 anaplastic carcinomas and 3 adenomas. Adenomatoid alveolar proliferation was frequently encountered. Adenosquamous lesions, composed of squamoid cells in a glandular pattern reminiscent of that seen in the tracheal papillomas, were observed in 2 animals.

Table 2. Number of survivals in weeks (% between brackets) of Syrian golden hamsters, treated with B(a)P/saline control

Group	Total No. of animals	Effective No. of animals	% of survivals in weeks									
			0	10	20	30	40	50	60	70	80	90
1	48	47	48(100)	46(95)	41(85)	33(68)	20(41)	10(20)	3(6)	2(4)	0	0
2	48	46	48(100)	48(100)	48(100)	47(98)	40(83)	29(60)	18(37)	7(14)	3(6)	0
3	48	46	48(100)	43(90)	39(81)	37(77)	27(56)	18(38)	6(13)	2(4)	0	0

Table 3. Tumor incidences in Syrian golden hamsters treated with B(a)P/saline and saline

Group	Treatment	Effective No. of animals	Total No. of respiratory TBA*	Total No. of respiratory tumors	Larynx		Trachea			Bronchi & Lung			Other tumor bearing animals
					Papilloma	Squamous cell carcinoma	Papilloma	Squamous cell carcinoma	Other tracheal tumors	Papilloma	Squamous cell carcinoma	Adenoma	Anaplastic carcinoma	
1	Large particles of B(a)P	47	31	55	5	—	12	20	2	2	9	3	2	16†
2	Small particles of B(a)P	46	5	6	1	1	4	—	—	—	—	—	—	17†
3	Saline	—	—	—	—	—	—	—	—	—	—	—	—	—

*TBA = tumour bearing animals.
†Forestomach papillomas.

Interstitial fibrosis was minimal, with infrequent accumulation of alveolar macrophages and pulmonary edema. In addition to the respiratory tumors, there were 16 forestomach papillomas.

Administration of small B(a)P particles (group 2) induced (in only 5 animals) neoplastic alterations, consisting of 1 papilloma, 1 squamous cell laryngeal carcinoma and 4 tracheal papillomas, in addition to an adenosquamous lesion similar to that seen in the previous group. No squamous cell carcinomas were observed in either the trachea or lungs. The pulmonary response in group 2 was otherwise minimal, and fibrosis or granulomas were not observed. Terminal alveolar congestion and edema were encountered occasionally. In addition to the lung tumors, there were 17 forestomach papillomas. The saline control (group 3) presented no respiratory tumors or lesions. Nephropathy with glomerular cellular proliferation and scarring was a common cause of death. Pulmonary congestion and infectious changes were infrequent.

The results of the retention rate study show a definite correlation between particle size and the rate of carcinogen clearance from the lungs,

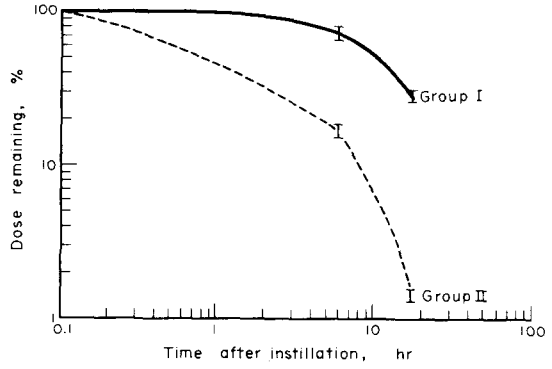

Fig. 1 Retention rate of B(a)P. Group 1—large particle size. Group 2—small particle size.

as shown in Fig. 1. A single dose, consisting of 2.96 mg of small particles of B(a)P in suspension, was cleared rapidly from the lungs; 17% was recovered after 6 hr and 1% after 18 hr. In contrast, the large particle B(a)P suspension was retained in the lungs for a longer period, with 75% recovered after 6 hr and 25% after 18 hr. No B(a)P of either particle size was recovered after 3 days. Thus, the large particles retarded the clearance of the carcinogen from the hamster lungs. Morphologically detectable alterations, specifically associated with particle size in the retention rate study, were not observed. Tracheobronchial epithelial desquamation, ciliacytophtoria, slit formation and alveolar cellular proliferation were seen in both carcinogen-treated groups. Saline instillation failed to produce any changes in the respiratory system.

DISCUSSION

Our results clearly indicate a decisive influence of particle size in experimental respiratory tumor induction. In early studies B(a)P failed to induce tumors when the carcinogen was instilled either alone [1] or in fine particles [4]. In the latter case when B(a)P was instilled in gelatin–saline suspension without a carrier dust, tumors occurred only at higher dose levels (about 100 mg) [3]. The first report on tumor induction by B(a)P alone dealt with instillation of large particles of the carcinogen (only 19% less than 10 μm and as much as 28% above 30 μm) in saline suspension [5]; here, the large B(a)P particles may have acted both as "carrier" particles and as a carcinogen. A positive dose–response relationship was demonstrated when low B(a)P doses were instilled for a longer time period [9].

Morphologically, the tumors in the present study were similar to those in man [10], consisting of mostly tracheobronchial surface papillomas and squamous cell carcinomas, preceded by epithelial metaplastic and dysplastic changes. The glandular tumor incidence was small: 5.5% in group 1, or nonexistent in group 2. The results are similar to those previously described [5, 9], pointing to a role of squamous metaplasia in respiratory tumor formation, and an association between polycyclic hydrocarbon inhalation and an increase in squamous cell carcinoma in man [11]. Morphological analysis of early B(a)P-induced lesions have also shown squamous metaplasia and atypia to be closely associated with subsequent tumor formation [10]. B(a)P-induced tumors occurred in a high incidence in the trachea (62%), compared to the larynx (9%) and lungs (29%) (group 1). A possible explanation is that the large particles (group 1) (64% above 10 μm) are propelled by the tracheobronchial surface mucociliary mechanisms in a proximal direction, which has less effect on smaller particles (group 2) (98% less than 10 μm). The lack of fibrogenic and granulomatous response in this study also may be due partially to the particle size.

An increased tumor incidence caused by adding carrier dust has been considered as being due to increased B(a)P retention [1, 12]. This aspect was also studied using the same B(a)P suspension. Large B(a)P particles were retained in the lungs for a longer time (25% recovered

after 18 hr) (Fig. 1), while the smaller particles cleared at a much faster rate (1% recovered after 18 hr). This tends to agree with previous reports that smaller particles are removed up to 95% in 5 hr [3], while larger particles are cleared only up to 50% in 24 hr [5]. These results, along with those in previous reports, suggest that large particles of B(a)P are retained longer in the lungs, acting both as carcinogen and "carrier" particles, while small B(a)P particles act only as a carcinogen and clear faster from the lungs, thus exhibiting only a very low level of carcinogenicity.

Previous studies have indicated an association between particle size, retention rate and subsequent respiratory tumor formation [13–19]. Saffiotti [20] reported results from a study using 3 different B(a)P samples, one prepared by grinding (particles up to 8.2 μm), one by precipitation (particles up to 17.5 μm) and one similarly prepared by ultrasonic treatment (particles up to 8.2 μm). The results indicated that the percentage of B(a)P retention decreased with decreasing particle size, as shown in this study. These results must be considered when comparing respiratory tumorigenesis studies, and the significance of different etiological agents. The particle size must also be considered in evaluating environmental and occupational hazards from respiratory cancer-inducing agents.

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The Current Causes of Death in Patients with Malignant Melanoma

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Abstract—*The immediate cause of death was determined for all patients dying at Memorial Hospital from* 1973 *to* 1976 *with biopsy-proven malignant melanoma. One-third of patients died of a central nervous system involvement. Pulmonary involvement and infections were the next most common causes of death. The addition of chemotherapy or chemoimmunotherapy to surgery did not change the causes of death. The incidence of postmortem, documented CNS metastases has also increased since prior studies* 10 yr *ago.*

INTRODUCTION

MALIGNANT melanoma is a tumor of interest as its incidence is increasing at a rate greater than any other tumor except lung cancer [1, 2]. Recent studies have suggested that both chemotherapy and chemoimmunotherapy have at most limited effects in this neoplasm [3–5]. As the immediate cause of death in melanoma patients has not been carefully examined since the advent of chemotherapy and chemoimmunotherapy, this study examined the effect of current therapeutic modalities on the cause of death in these patients.

MATERIAL AND METHODS

The immediate cause of death in all patients with biopsy-proven malignant melanoma dying at Memorial Hospital between 1973 and 1976 were examined. Patients treated at Memorial but dying elsewhere were excluded from study to ensure uniformity of both medical care and pathologic findings during the final admission. Approximately 50% of patients followed for malignant melanoma at Memorial Hospital were included in this study. A total of 139 patients were evaluated. The clinical cause of death was determined by using the criteria of a previous study on the causes of death in cancer patients [6]. A CNS catastrophe was defined as a primary neurologic event due to tumor leading directly to the patient's demise. Intracranial hemorrhage, herniation and/or progressive metastatic disease with irreversible neurologic signs leading to death were included under this category. Infectious causes required cultures and/or evidence of an infection such as fever with leukocytosis and/or a pulmonary infiltrate which led to respiratory and/or vasomotor collapse not explainable by other causes. Respiratory causes of death only included pulmonary insufficiency directly caused by the metastatic process and not by superimposed infection. A hemorrhagic cause of death was defined as uncontrollable bleeding leading to vaso-motor collapse and/or leading to irreversible damage of a vital organ. Supportive autopsy evidence was available in 73 patients. When the immediate cause of death was clinically or pathologically uncertain the patient was scored as having "other" cause of death.

The age at time of death, sex and stage of disease at first diagnosis were determined. Final laboratory values, if within two days of death, were noted. The patients were then placed into one of three groups (Table 1) according to the type of treatment they received:

(1) Surgery and/or irradiation.
(2) Chemotherapy in addition to surgery and/or radiation therapy. Chemotherapy usually involved treatment with nitrosurea and DTIC initially, followed by multiple other experimental agents.
(3) Immunotherapy, in addition to surgery and/or radiation therapy plus chemotherapy. Thirty-five patients received only BCG, 10 *C. parvum*, 5 mixed bacterial vaccine (a variant of Coley's toxins), 3 allogeneic tumor vaccine and 2 transfer factor.

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Table 1. *Characteristics of patients studied*

	Group 1 (surgery)	3 (+chemotherapy)	3 (+immunotherapy)	Total group
Number of patients	24	69	46	139
Male/female	18/6	38/31	29/17	85/54
Medain age	63	48	50	52
(range)	(32–92)	(20–76)	(16–73)	(16–92)
Stage, at time of diagnosis of melanoma				
Localized (skin ± nodes)				
Number of patients	19	51	41	111
Disseminated	5	14	4	23
Not determined		4	1	5
Second malignancies in patients	4	2	3	
	1. Breast cancer 2. Cervical carcinoma 3. Adenocarcinoma of the sigmoid colon 4. Adenocarcinoma of the sigmoid colon, lymphoma	1. Breast cancer 2. Adenocarcinoma of the sigmoid colon	1. Thyroid papillary adenocarcinoma 2. Adenocarcinoma of the colon 3. Lymphoma, Basal cell carcinoma of the skin	

The causes of death were then examined as indicated above and statistical analysis was done by chi square analysis with Yates' correction.

RESULTS

Table 1 indicates the composition of the patients studied. No significant differences among the three groups were found. Males predominated in all groups. Approximately 80% of patients in each group had localized skin or localized skin with proximal node metastases at the time of initial presentation.

Table 2 indicates the final hematologic values at time of death. Patients treated with either chemotherapy or chemoimmunotherapy tended to be more anemic, have a higher BUN and lower platelet count than patients treated solely with surgery and/or radiation. These differences were not of statistical significance. Hypercalcemia, defined as a serum calcium greater than 11.0 mg per deciliter, was noted in three of the chemotherapy and three of the chemoimmunotherapy patients.

Table 2. *Laboratory values of study patients*

	Percentage of abnormal values at time of death		
	Group 1	Group 2	Group 3
Hemoglobin < 10.0	35	45	54
White blood cell count < 2.0	5	6	5
Platelets < 100,000	15	21	25
BUN > 25	29	49	54

The most common causes of death (Fig. 1) were (1) central nervous system involvement by tumor ($P < 0.03$ compared to pulmonary involvement), (2) metastatic pulmonary involvement with respiratory insufficiency and (3) infections. One-half of the infectious processes in each group were pneumonias. Therapy did not change the relative frequency of the various causes of death in any of the groups (Fig. 1).

The extent of metastatic disease found on autopsy is shown in Table 3. Patients dying without evidence of melanoma (2 patients) or in the immediate postoperative period (3 patients) were excluded from analysis. Except for central

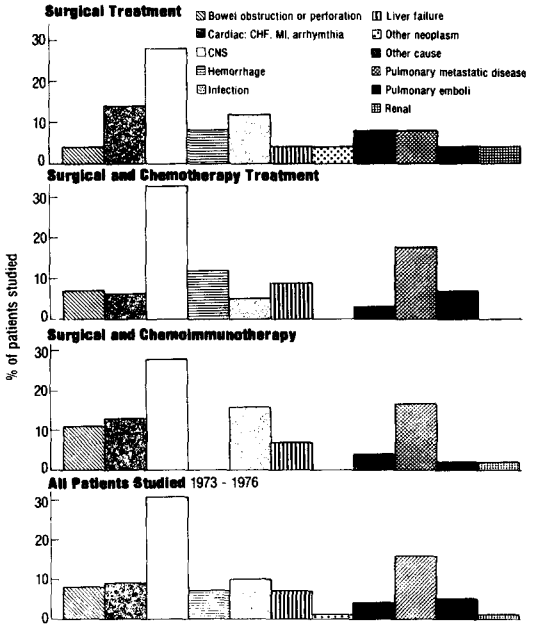

Fig. 1. *Causes of death in melanoma patients.*

Table 3. Extent of disease proven by autopsy
Percentage of involvement by organ system

	Group 1	Group 2	Group 3	Total	Historical control Memorial Hospital [7]
Number of patients	8	35	24	67	125
Median age	58	47	45		
	Site of metastasis Percentage of involvement by organ system				
Bladder and ureter	0	23	29	22	18
Bone	25	57	71	58	Not stated
CNS	88*	69*	75*	73*	39
Kidney	63	34	50	45	45
Heart	50	51	54	52	49
Liver	63	71	71	70	68
Lung	88	77	75	78	70
Stomach and intestines	50	66	75	67	58

*Different from historical control, $P < 0.05$.

nervous system (CNS) involvement, the sites of involvement at time of death has not changed compared to historical controls at Memorial Hospital [7]. Approximately three-quarters of all study patients had CNS involvement at time of death (Table 3) which is almost twice the frequency previously reported from this Institution. CNS involvement usually demonstrated multiple mass lesions in the brain.

The total percentage of second malignant tumors in the patients studied was 6%. Four patients in the Group 1, two in Group 2, and three in Group 3 had at least one other malignancy (Table 1). No predominance of specific pathologic type of neoplasm was noted.

DISCUSSION

Several recent reviews have stressed the importance of infection in causing the demise of patients with cancer [6, 8]. However, these studies contained a heterogenous group of malignancies. Based upon these findings we have examined the immediate cause of death in one specific neoplasm and have noted that infection is not the major cause of death.

Approximately one-third of all patients with malignant melanoma die of CNS involvement independent of the modalities of treatment used. A previous report has emphasized that all patients with pathologic evidence of CNS involvement at autopsy died of CNS involvement [3]. This study did not confirm this finding as 73% of patients had pathologic proof of CNS metastases (Table 3), but slightly less than one-half that number died as a result. Since the last review of autopsy material from this Institution, however, the incidence of CNS involvement seems to have markedly increased. Patients in the current study had almost twice the frequency of involvement as did patients examined a decade ago at Memorial Hospital (Table 3). The reasons for this apparent increase are obscure, but may reflect more aggresive supportive care of other organ system involvement thus allowing a longer survival time to make neurologic disease evident. There is no change in the method of pathologic examination of the central nervous system since the last study to account for this finding.

Pulmonary insufficiency caused by metastatic disease in the lungs was the second most common cause of death in the total group of patients. This cause of death has not been previously stressed. Infection was the third most common cause of death. As pneumonia accounted for one-half of infectious causes of death, a pulmonary demise whether secondary to metastatic disease or infection was very frequent. Heightened awareness of possible infectious complications and early use of antibiotics may account for the lowered incidence of infections seen in this study compared to previous reports. In addition, we did not find the severe neutropenia seen in a previous study [8]. Whether this difference represents less aggresive cytotoxic therapy or heightened supportive care to maintain the patient over his leukopenic episode remains uncertain.

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Effects of Ehrlich Ascites Tumor Transplantation in Mice on the Distribution of Cells Capable of Forming Hemopoietic Colonies *in vitro*

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Abstract—*The effects of intraperitoneal Ehrlich ascites tumor development in CBA/H-T_6 mice on the distribution of hemopoietic colony forming cells in the host bone marrow and spleen were studied using soft agar culture techniques. Cell suspensions of hemopoietic tissues obtained from mice at various days after i.p. transplantation of* 10^6 *tumor cells as well as from control mice were cultured for* 7 days *to grow granulocyte/macrophage colonies (CFU-C) in the presence of standard colony stimulating activity (CSA) provided by serum of endotoxin-treated mice and B lymphoid colonies in the presence of 2-mercaptoethanol. The CFU-C level in the femoral marrow of tumor-bearing mice declined rapidly both in concentration and absolute number to* 36 *and* 17% *of control values, respectively, between* 3–5 days, *followed by a gradual recovery to normal by* day 9. *In contrast, these values increased continuously in the spleen to* 27- *and* 75-*fold normal, respectively, by* 9 days. *B lymphoid colony-forming cells increased in concentration and absolute number in the spleen to* 5- *and* 10-*fold normal values, respectively, at* 7 days. *High levels of CSA, which promoted CFU-C development when added to normal marrow cells, were detected in the cell-free Ehrlich ascites serum. When either tumor cells or host-derived leukocytes fractionated from the tumor were used as feeder layers, major stimulation of CFU-C growth in normal marrow was seen with the former, and some minor stimulation with the latter. These* in vitro *studies, combined with our previous studies* in vivo *on spleen colony forming units (CFU-S), suggest that Ehrlich ascites tumor transplanted in mice stimulates a rapid redistribution of hemopoietic stem cells at various levels of differentiation from marrow to spleen, where they may undergo further growth; the stimulus is possibly mediated by factors liberated from the tumor.*

INTRODUCTION

A SERIES of investigations was undertaken in this laboratory to elucidate the overall response of the host lympho-myeloid system to heterotopic tumor transplantation in mice. These included an evaluation of the life history of host leukocytes migrating into the tumors [1, 2], an examination of the surface markers of lymphocytes accumulating in the tumors and host lymphoid organs [3, 4] and hemopoietic changes in the tumor bearing animals [5, 6]. The last named study revealed that intraperitoneal transplantation of Ehrlich ascites tumor (EAT) or TA-3(St) tumor in mice caused a rapid decline in the incidence as well as absolute number of pluripotent hemopoietic stem cells (spleen colony forming units, CFU-S) in the femoral marrow, associated with a rapid rise of these values in the spleen, and to a small extent in the blood. This suggested an accelerated CFU-S traffic from the host marrow to the spleen, where an active proliferation of this cell class was identified from ^{3}H-thymidine suicide experiments. However, one could not exclude the remote possibility that the observed changes in the spleen colony-forming potentials of the marrow and the spleen reflected a differential alteration in the homing properties of stem cells in these organs rather than a redistribution phenomenon. It was felt that *in vitro* colony assays could resolve this issue and in addition, reveal (1) whether tumor transplantation affected the distribution of "committed" stem cells, and (2) whether the tumors elaborated materials capable of stimula-

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ting hemopoiesis. The study using soft agar cultures was therefore designed to examine (1) the effects of EAT transplantation on the organ distribution of cells capable of developing into granulocyte-macrophage colonies (CFU-C, considered as partially differentiated progeny of CFU-S) and (2) the possible elaboration of colony (CFU-C) stimulating activity (CSA) by the EAT. The presence of CSA was examined in the cell free Ehrlich ascites serum or blood serum of tumor bearing animals when added directly to cultures of normal bone marrow, as well as in the various cellular fractions of EAT when provided as feeder layers. Furthermore, since the spleens of these tumor bearing hosts showed an increased incidence of newly-formed, marrow-derived small lymphocytes [5, 6], the present study also evaluated in these spleens the incidence of cells capable of producing B lymphocyte colonies on agar in the presence of 2-mercaptoethanol [7]. These investigations revealed a major redistribution of CFU-C from the host marrow to the spleen—a finding which reinforced the conclusion of CFU-S redistribution, an elaboration of high levels of CSA by the tumor cells and an increase in the number of B lymphocyte colony forming cells within the host spleens.

MATERIAL AND METHODS

Tumor-host system

Ehrlich ascites tumor (EAT) used in the present study was maintained in inbred CBA/H-T_6 female mice (Jackson Laboratories, Bar Harbor, Maine) at 12–16 weeks of age by a weekly intraperitoneal transplantation of 10^6 tumor cells in 0.1 ml isotonic saline. This tumor was started as a subline 4 yr ago by transferring 10^6 tumor cells from our conventional EAT line maintained in CF_1 female mice into CBA/H-T_6 strain mice. The modal chromosome number of tumor cells is 68, and post-mitotic tumor cells contain a near tetraploid amount of DNA. Following an i.p. transplantation of 10^6 cells, tumor growth is exponential during the first 3 days, and a plateau population size of approximately 400 million tumor cells is attained by 7 days. The median survival time of tumor bearing animals is approximately 2 weeks.

Sampling of hemopoietic tissues for culture on soft agar

Groups of animals were killed by cervical dislocation, and the femora and spleens removed under aseptic conditions both from normal and tumor bearing hosts at various intervals after tumor transplantation. Femora were cleaned with gauze pads and the epiphyseal ends were carefully removed to expose medullary cavities which were then flushed out with bone marrow collecting fluid described by Metcalf and Moore [8] using a 23 gauge needle fitted to a sterile syringe. The extruded marrow was dispersed by gentle aspiration with a syringe through an 18 gauge needle. Spleens were minced in bone marrow collecting fluid with a pair of deWecker's iris scissors and gently aspirated with a sterile syringe through an 18 gauge needle. Marrow and splenic cell suspensions were freed of stroma and vessels by passage through a stainless steel sieve (80 mesh, 0.005 gauge) followed by 2 washes in bone marrow collecting fluid by centrifugation at 400 ***g*** for 10 min. The final pellets were resuspended, mixed well with a vortex mixer and the numbers of nucleated cells in the resulting single cell suspensions were determined using a particle counter (Coulter Electronics, Model B). Cells were kept on ice at 4°C until used for plating shortly after collection.

Soft agar culture

Aliquots of 1 ml 0.3% agar-medium containing the appropriate cell concentrations were pipetted into 35 mm plastic Petri dishes [8], allowed to gel at room temperature, and placed in a specially designed plexiglass incubating chamber. Colony stimulation was provided by a number of sources, to be described.

Incubation and scoring of cultures

Cultures were all incubated for 7 days in a humidified atmosphere of 10% CO_2 in air maintained at 37°C. Colonies (aggregates of 40 or more cells) and when feasible, clusters (small, 10–19 cells and large, 20–39 cells) were counted with a dissecting microscope (Zeiss) at 40 × magnification with indirect lighting. Squash preparations of agar cultures were prepared and stained with aceto-orcein for closer morphological examination. In addition, selected individual colonies from 2-mercaptoethanol stimulated cultures were transferred with fine-tipped Pasteur pipettes to microscope slides, air dried, methanol fixed and stained with Giemsa (pH 5.75).

Preparation of colony stimulating activity (CSA) sources for promoting CFU-C growth

(a) Post-endotoxin serum was prepared as a standard source of CSA [9] to evaluate changes in the number of CFU-C in bone marrow and spleen during tumor development. Adult female A/HeJ mice (Jackson Lab) were injected i.p. with 5 μg (in 0.1 ml vol) of *Salmonella typhosa* endotoxin (Difco) and after 2–4 hr their blood

was collected by cardiac puncture under light ether anesthesia and allowed to clot. Following centrifugation at 600 ***g*** for 20 min, the cell-free serum was treated by dialysis and centrifugation [10] to remove lipoproteins and dialyzable inhibitors of colony formation. The serum was then Millipore filtered (0.2 μm pore size) and stored in 1 ml aliquots at $-20°C$ until used.

(b) Cell-free Ehrlich ascites serum (from 7 day old tumors) and peripheral blood serum from mice bearing 7 day old tumors were collected and similarly treated prior to storage, as described for post-endotoxin serum. These sera were assayed for their CSA content as compared to that in the post-endotoxin serum on cultures of 10^5 nucleated marrow cells.

(c) Serum-free 7 day old ascites tumor and its various cellular fractions were tested as possible CSA-sources when provided as feeder layers. Ascites tumors were harvested without anticoagulant treatment and treated briefly with 0.83% ammonium chloride solution for lysis of erythrocytes [11]. This provided total nucleated cells which were washed and resuspended in bone marrow collecting fluid. To obtain leukocyte-rich or tumor cell-rich fractions from this nucleated cell population, modification of a sucrose density gradient technique [12] was employed. Briefly, suspensions of 60×10^6 nucleated cells/ml of Hanks' balanced salt solution (BSS) were layered in 1 ml vol onto 14 ml linear gradients of sucrose (5–15% in Hanks' BSS containing 20% fetal calf serum throughout). After gentle centrifugation (100 ***g*** for 10 min) the supernatants containing mostly leukocytes and pellets containing mostly tumor cells, were collected separately, washed twice in an excess of Hanks' BSS and then washed and resuspended in bone marrow collecting fluid.

Feeder layers containing 5×10^5 nucleated cells (from sources described above) in 1 ml 0.5% agar-medium were added to culture plates and allowed to gel prior to the addition of 1 ml 0.3% agar medium containing 10^5 nucleated marrow cells. After plating, smears were made from the remaining cell suspensions, stained with MacNeal's tetrachrome blood stain and differential counts were performed.

Determination of optimal concentration of post-endotoxin serum used as standard source of CSA

The following volumes of post-endotoxin serum were added to culture dishes prior to the addition of 1 ml aliquots of 0.3% agar medium containing a standard number of 10^5 nucleated marrow cells: 0.05 ml; 0.10 ml; 0.15 ml. Triplicates were made of each plate and colonies were scored after 7 days incubation. The increase in the number of colonies was nearly proportional to the concentration of post-endotoxin serum. Thus, 0.05 ml, 1.0 ml and 1.5 ml of this serum stimulated the production of 39.6 ± 1.5 (mean $\pm$ S.E.), 69.0 ± 2.6 and 100 ± 3.6 colonies, respectively. Since there was some colony confluence in the last case, 0.1 ml of post-endotoxin serum was selected as the optimal dose for providing CSA throughout this present study.

Determination of optimal cell numbers for plating in CFU-C studies

In pilot studies, a 0.1 ml vol of post-endotoxin serum was included in culture plates containing graded numbers of nucleated marrow and splenic cells. Triplicates were made of each plate and colonies were scored after 7 days incubation. As shown in Table 1, colony numbers increased in a linear fashion with increasing cell concentrations in culture. However, normal splenic cells produced poor colony numbers even at very high doses. In contrast, at comparably high doses of splenic cells from tumor bearing animals, colony-confluence led to scoring difficulties. To avoid this problem, optimal nucleated cell number for plating was considered as 2.5×10^5 for spleens and 10^5 for bone marrow.

Table 1. Colony (CFU-C) numbers (mean $\pm$ S.E.) produced by different doses of marrow and spleen cells in the presence of 0.1 ml *post-endotoxin serum*

Control bone marrow Nucleated cell dose ($\times 10^5$)	Spleen	
	Control mice	7 day EAT host
0.5	34 ± 3	—	—
1.0	69 ± 2.6	—	—
2.5	280 ± 25	2.0 ± 0.6	45 ± 5
5	—	4.5 ± 1	105 ± 4
10	—	10 ± 1.6	223 ± 9

2-mercaptoethanol stimulation of splenic cultures for B lymphocyte colony formation

Splenic cell suspensions from normal and tumor bearing mice were prepared and added to 0.3% agar medium, as described previously. One ml aliquots containing 10^6 splenic cells were pipetted into Petri dishes containing 2-mercaptoethanol at a final concentration of 5×10^{-5} M[7]. Cultures were scored for B lymphocyte colonies and clusters after 7 days of incubation.

RESULTS

Marrow and splenic CFU-C levels during tumor development

Bone marrow. Table 2 shows the number of colonies and clusters per 10^5 nucleated marrow cells at various intervals after tumor transplantation. Data on colonies are plotted in Fig. 1, expressed also as fractions of the incidence in the control marrow. The CFU-C concentration in marrow declined drastically after tumor transplantation, reaching levels 35–42% of control values between days 3 and 7, followed by a rapid recovery to control level by day 9. Since bone marrow cellularity also declined during the early intervals of tumor development (Table 3), the changes in the absolute number of CFU-C per femur were found to be more dramatic (Fig. 2). For example, this value declined rapidly from 5980 at 0 day to a minimal level of 1011 (approximately 17% of control) by day 3, followed by a gradual recovery to near normal level by day 9 (5,228 CFU-C, 87% of control).

Spleen. Colony and cluster numbers per 2.5×10^5 nucleated splenic cells are presented in Table 2. Following a short lag of 3 days, the CFU-C concentration in the spleen increased rapidly and by day 9 there was some 27 fold magnification compared to the control level (Fig. 3). These data became even more significant when the splenic enlargement after tumor transplantation (Table 3) was considered. The splenic CFU-C content (Fig. 4) appeared to increase without any lag; however, the rise was steepest between days 3 and 5, giving a doubling

Table 2. Mean (±S.E.) incidences of clusters (20–39 cells) and colonies (≧40 cells) in the femoral marrow (per 10^5 nucleated cells) and spleen (per 2.5×10^5 nucleated cells) at different days of tumor development, when cultured in the presence of post-endotoxin serum*

Day	Clusters		Colonies	
	Marrow	Spleen	Marrow	Spleen
0	38.6±7.9	1.0±1.0	52.00±8.1	2.0±0.6
3	7.5±3.2	4.3±1.7	21.50±4.7	4.3±1.7
5	24.8±2.8	3.0±0.7	18.30±2.5	15.5±1.4
7	20.8±3.9	5.8±2.2	22.01±1.9	39.5±3.8
9	n.d.†	n.d†	61.7 ±2.7	53.0±2.3

* Of 4 replicate cultures in each case.
† Not determined because of high colony numbers interfering with the accuracy of cluster counts.

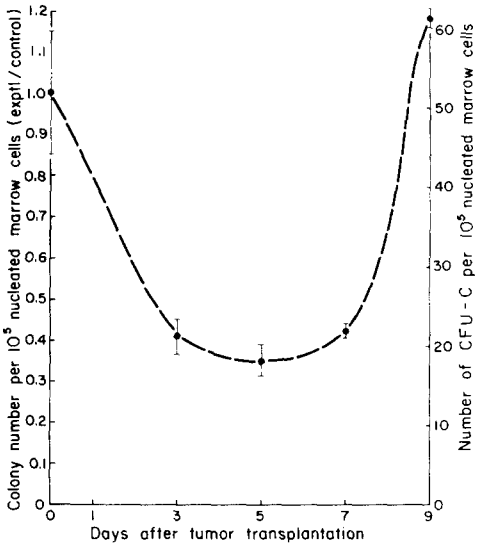

Fig. 1. Temporal changes in the incidence of CFU-C in femoral marrow (expressed as number per 10^5 nucleated cells, as well as fraction of the value in control animals).

Table 3. Nucleated cell content of femoral marrow and spleen at different days of tumor growth

Day	Cell number × $^{-6}$ (mean±S.E.)	
	Femur	Spleen
0	11.5±1.1	61.7±5.2
3	4.7±1.4	92.0±8.5
5	12.3±0.7	124.0±10.2
7	10.7±1.0	127.6±12.1
9	8.5±0.9	176.3±19.4

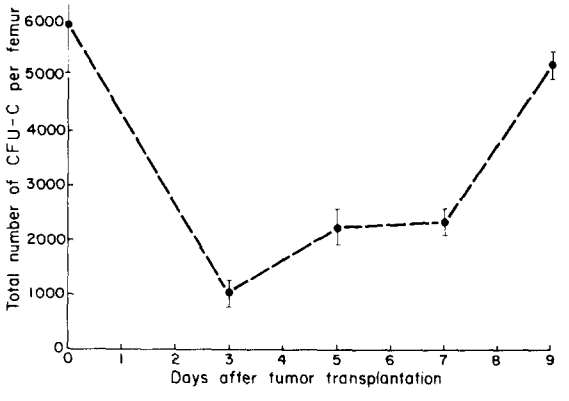

Fig. 2. Absolute number of CFU-C per femoral marrow at different days of tumor development.

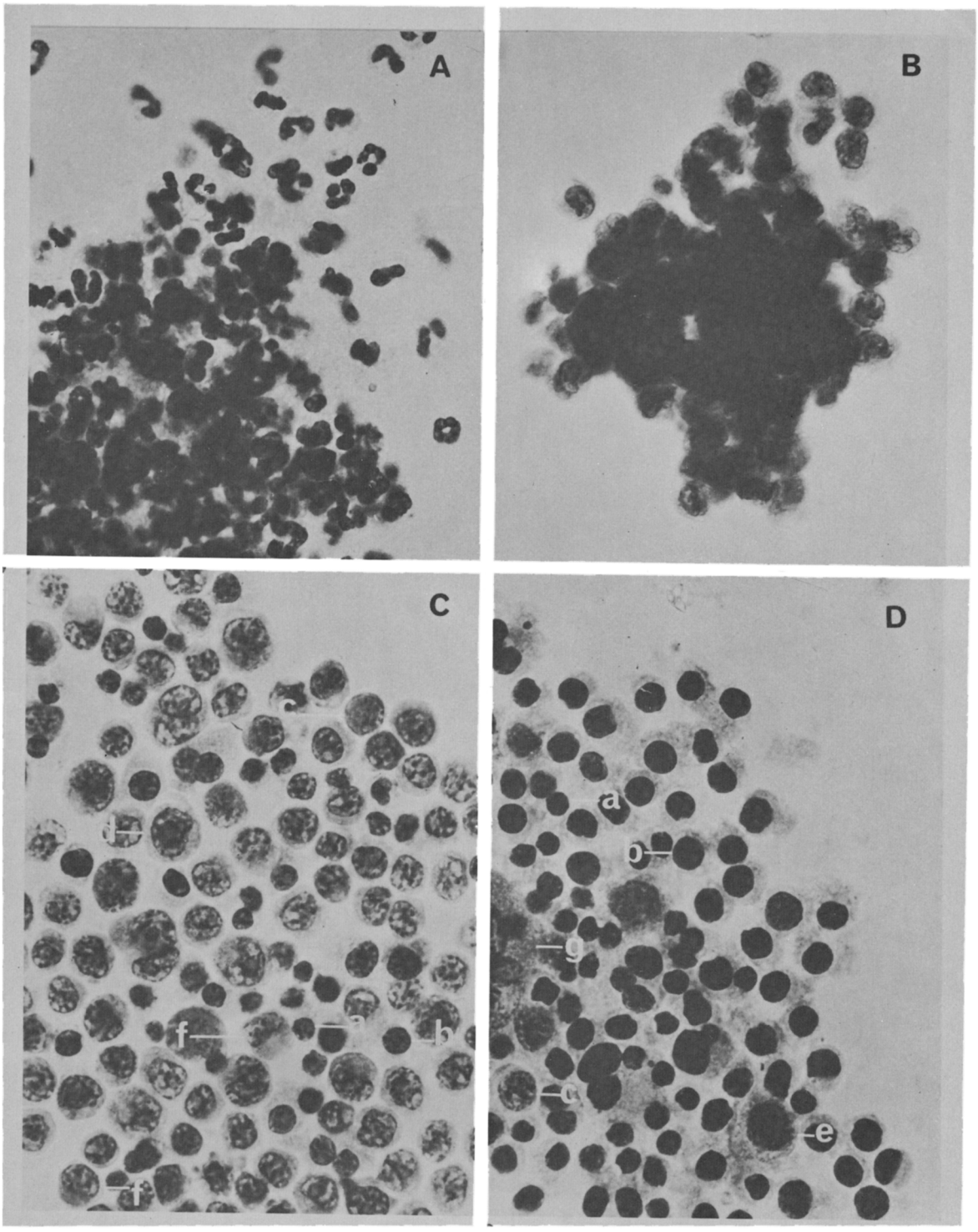

Fig. 5A. Portion of a typical, compact granulocytic colony produced by the spleen at 5 days *of tumor growth. Mature segmented granulocytes are seen at the periphery and less mature cells are located towards the centre.* (×1320).

Fig. 5B. A compact granulocytic colony produced by normal bon marrow in the presence of a feeder layer containing Ehrlich ascite tumor cells. In contrast to Fig. 5A, peripheral cells are less mature, showing only early signs of nuclear lobulation in some cases (×1320).

Figs. 5C and D. Portions of B lymphocyte colonies produced by splenic cells at 3 days (*Fig. 5C*) *and* 7 days (*Fig. 5D*) *of tumor growth. Lymphoid cells of all types are seen within these colonies: small lymphocyte* (*a*), *medium lymphocyte* (*b*), *large lymphocyte* (*c*), *blast cell* (*d*), *blast cell in mitosis* (*e*) *and immature plasma cell* (*f*). *Part of a megakaryocyte* (*g*) *is seen in the central part of the colony in Fig. 5D.* (×1320).

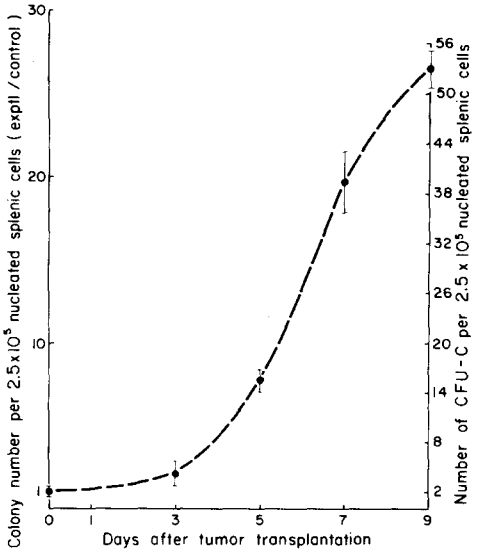

Fig. 3. Temporal changes in the CFU-C concentration in the spleen (expressed as number per 2.5×10^5 *nucleated cells as well as ratio to control values).*

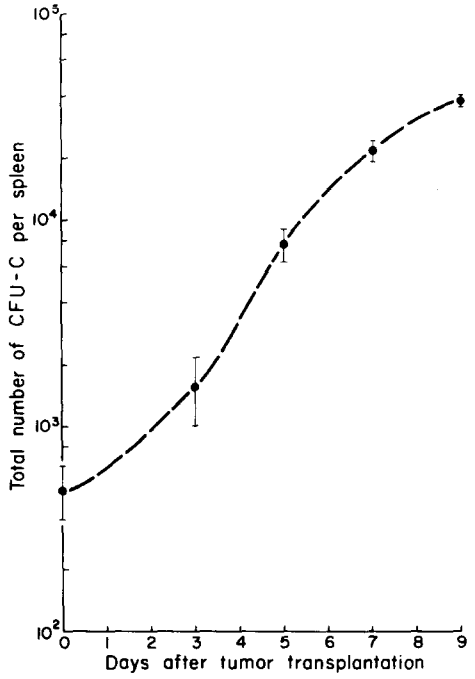

Fig. 4. Absolute number of CFU-C per spleen at different days of tumor development.

time of approximately 1 day during this interval. By day 9, the CFU-C number increased to approximately 75 fold of control values.

CFU-C morphology. Colonies were either compact or dispersed in appearance. Compact colonies were predominantly granulocytic in nature, often showing the whole range of maturation sequence starting from the center to the periphery (Fig. 5A). Dispersed colonies contained either granulocytes, or macrophages or a mixture of both cell classes. The presence of a tumor did not seem to influence the composition of colonies derived from any organ. However, cultures of spleens showed a preponderance of granulocytic colonies compared to the bone marrow cultures. For example, the average incidences ($\pm$S.E.) of colony types in normal splenic cell cultures were: granulocytic $83 \pm 8.3\%$, macrophage $8 \pm 2.5\%$ and mixed $9 \pm 2.8\%$; for normal bone marrow they were $45 \pm 7\%$, $37 \pm 6.1\%$, and $18 \pm 3\%$ respectively.

Evaluation of colony-stimulating activity (CSA) in sera from ascites tumors and peripheral blood of tumor bearing hosts

The CSA of various sera, in 0.1 ml vol, was assayed on 10^5 nucleated marrow cells and the results are presented in Fig. 6. Sera from the

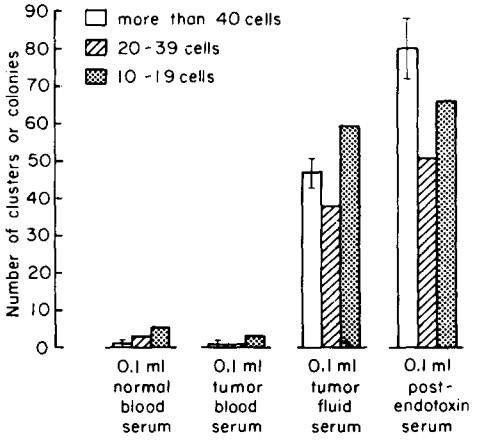

Fig. 6. Comparison of cluster or colony (CFU-C) numbers produced by 10^5 *normal bone marrow cells in the presence of various sera.*

peripheral blood of both normal and 7 day ascites tumor bearing mice exhibited no significant CSA, producing only 1.0 ± 0.6 and 0.3 ± 0.3 colonies. However, 7 day Ehrlich ascites exhibited significant CSA, producing 47 ± 3 colonies, compared to post endotoxin serum which stimulated the production of 80 ± 8 colonies. In addition, both these latter sera resulted in the production of many clusters. Since no activity was detectable in the blood sera of tumor-bearing animals, local production of CSA within the tumor was indicated.

Colony morphology was similar to that described in the previous section, with no significant differences observed in the colony types produced by stimulation with various sera.

Evaluation of various cellular fractions of Ehrlich ascites tumor as possible sources of CSA when used as feeder layers

The composition of various cellular fractions of the 7-day ascites tumors used in this assay is shown in Table 4. The degree of colony stimulation provided by these fractions to cultures of 10^5 nucleated marrow cells is indicated in Fig. 7. Feeder layers containing 5×10^5 cells of the leukocyte rich fraction

Table 4. Incidence (percent) of various cell types in the 7 day old ascites tumor and its various cellular fractions used in feeder layers as possible sources of CSA

	Tumor cells	Total leukocytes	Lymphocytes	Granulocytes	Monocytes and macrophages
Unfractionated tumor	88.8	11.2	4.3	2.8	4.1
Tumor cell-rich fraction	95.5	4.5	0.6	1.7	2.2
Leukocyte-rich fraction	4.2	95.8	31.4	18.3	46.1

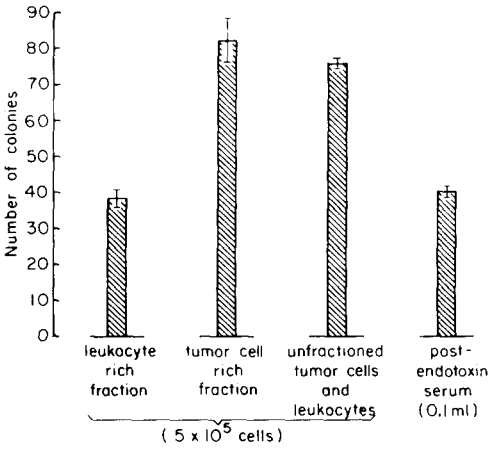

Fig. 7. Comparison of colony (CFU-C) numbers produced by 10^5 normal bone marrow cells grown in the presence of feeder layers containing various cellular fractions of 7 day old EAT.

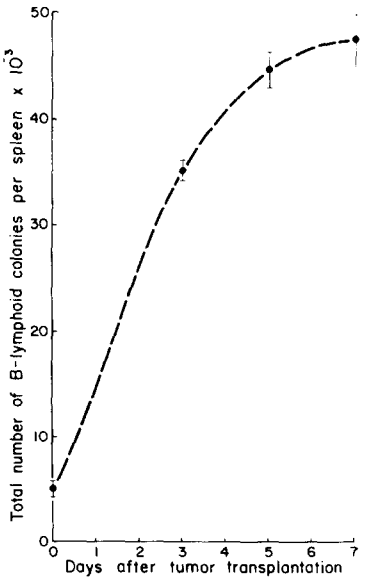

Fig. 8. Absolute number of B lymphocyte colonies per spleen at different days of tumor development.

stimulated the production of 39 ± 2 colonies, comparable to the 40 ± 1 colonies produced by 0.1 ml of post endotoxin serum within the same experiment. The tumor cell-rich fraction as well as the unfractionated tumor cells with their associated leukocytes provided a higher stimulation, producing 82 ± 6 and 75 ± 1 colonies, respectively. The difference between the latter two cases was not statistically significant at the 0.05 level.

Colony morphology. Colonies produced by stimulation with all three types of feeder layers were predominantly granulocytic in morphology, whether compact or diffuse in form, outnumbering macrophage colonies approximately 8–1. Mitoses were frequently seen in these colonies. Furthermore, in cultures stimulated by cells of the unfractionated tumor (11.2% leukocytes) or the tumor cell-rich fraction (4.5% leukocytes), a small percentage of the granulocytic colonies (approximately 10%) appeared more immature than those usually seen throughout this study after 7 days of incubation (Fig. 5B). Most of the cells in these colonies contained nuclei that were round or oval, and only the most peripheral cells showed certain degrees of nuclear lobulation characteristic of more mature forms. This colony type was not seen in cultures stimulated either by feeder layers of the leukocyte-rich fraction or by post-endotoxin serum.

Cells within the feeder layers themselves at 7 days of culture consisted of viable as well as dying cells, but no evidence of active proliferation was observed amongst these cells at this interval.

Changes in B lymphocyte colony forming potential of spleens during tumor growth

Table 5 shows the numbers of clusters and colonies produced by 10^6 nucleated splenic cells from mice at various intervals after tumor transplantation, using 2-mercaptoethanol stimulation of cultures. Colony incidence sharply increased to approximately 5 fold of control level by day 3, with no further significant change up to 7 days. When translated into absolute number of colony forming cells in the spleen (Fig. 8), the increase was 7 fold by day 3, followed by some further rise to a near plateau of approximately 10 fold normal level by 7 days.

Table 5. Number (mean of 4 replicate cultures ± S.E.) of lymphoid clusters (3–39 cells) and colonies (≧40 cells) produced in the presence of 2-mercaptoethanol by 10^6 splenic cells at various days of tumor growth

Days	Clusters	Colonies
0	565.3±24.7	81.5±12.4
3	203.5±41.2	392.0±10.7
5	239.8± 5.5	359.5±15.4
7	383.8±34.0	373.5±20.2

Colony morphology. Normal splenic cells formed colonies which were generally dispersed in nature and the cellular background was extremely dense. These cultures also contained a very large number of clusters. In contrast, spleens from mice either 3, 5 or 7 days after tumor transplantation produced large numbers of compact colonies (illustrated in Figs. 5C and D, ranging in size from 40 to several hundred cells. All colonies and clusters were composed of a wide variety of lymphoid cells: small, medium and large lymphocytes, blast cells and immature plasma cells. In addition to lymphoid colonies, mercaptoethanol stimulated cultures also showed small but significant numbers of megakaryocytic colonies. They were composed of 2–6 giant cells with multiple or multilobulated nuclei and abundant cytoplasm showing smooth rounded projections. Their incidences were not high enough for a statistical evaluation of possible differences between the normal and tumor-bearing mice.

DISCUSSION

Intraperitoneal transplantation of Ehrlich ascites tumor in CBA/H-T_6 was found to produce differential effects on the incidence of CFU-C in the host bone marrow and spleen, in a manner qualitatively similar to that recorded for CFU-S using the same tumor-host combination [6]. This similarity suggests that our previous findings on CFU-S reflected actual changes in the organ distribution of stem cells rather than a differential alteration of their homing properties detected by the assay system. Although blood CFU-C levels were not determined in the present study, the inverse relationship between the early changes in CFU-C numbers in the marrow and the spleen can be best explained, at least in part, by a redistribution phenomenon, as noted with CFU-S in the present [6] as well as other [13] tumor-host combinations.

In addition to CFU-C migration, a local production of CFU-C within the spleen by the process of multiplication and differentiation of CFU-S can best explain the pronounced and continued elevation of splenic CFU-C content at later intervals of tumor growth. For example, while the quantitative changes in the CFU-S and CFU-C contents were identical for marrow at all intervals, splenic CFU-C content continued to rise markedly (viz. to 75 fold at 9 days) even after the splenic CFU-S content reached a near plateau level (of 8–10 fold normal between 4–9 days). This suggestion is consistent with our earlier observations [6] of high proliferative activity in the splenic CFU-S, as evidenced by high ^{3}H-thymidine suicide rate, at 4–5 days of tumor growth when the near maximal CFU-S number had already been attained in the spleen. Thus, this proliferation appears to be a step in differentiation rather than self-amplification. Whether the stimulus for such proliferation is contained in the family of molecules identified as CSA, remains a matter of conjecture.

A significant level of CSA in the ascites serum appeared to result from local rather than systemic elaboration, since peripheral blood serum of the same tumor bearing hosts did not exhibit any detectable increase in CSA level. Results of the feeder layer experiments showed that tumor cells themselves represented a major cellular source of CSA and the leukocytes (possibly monocytes and macrophages) within the ascites tumor also elaborated CSA. The observed differences in the degree of stimulation provided by these two cellular constituents of the tumor could either reflect real differences in their CSA outputs or the effects of the presence of a higher proportion of colony inhibiting cells [14], viz. mature granulocytes in the leukocyte-rich fraction (Table 4). Observation of a significant proportion of immature granulocytic colonies growing on tumor cell feeder layers, but not in the presence of other CSA sources, may indicate that CSA released by the tumor cells is either deficient in some maturation factors or contaminated by factors that can cause "maturation-arrest". We favour the former possibility since similar colonies were not observed in the presence of Ehrlich ascites serum. This colony type is also found in the presence of supernatants from Ehrlich ascites tumor cell cultures (Eridani, personal communication). The absence of detectable CSA in the peripheral blood of the present tumor-bearing mice does not necessarily exclude possible *in vivo* effects of CSA elaborated by the tumor, since this may be rapidly utilized from the circulation. Relationship of CSA production *in vitro* to granulocytopoietic effects *in vivo* has been observed for some tumors [15].

A number of studies have reported changes in the CFU-S [13, 16–19] or CFU-C [20] contents

of mouse hemopoietic organs in other tumor-host combinations. Bone marrow generally showed a variable depletion [13, 16, 18] which may represent an acute effect of rapidly growing tumors. Possible reasons for this depletion are migration, death or growth inhibition. Although there is definite evidence for an enhanced migration [6, 13] other possibilities cannot be excluded completely. The marrow, in most cases, appears to show a quick recovery. In some cases with longstanding tumors, femoral stem cell content may show an actual rise, as reported for CFU-S in mice bearing transplanted fibrosarcoma [19] and spontaneous mammary tumors [6], or for CFU-C in mice bearing transplanted mammary tumors [20]. An increase in the splenic stem cell content appears to be an universal phenomenon for all tumor-host systems, applicable both to CFU-S [6, 13, 17] and CFU-C [20]. Although no work has been done on the erythropoietic progenitor cells (CFU-E) in tumor-bearing hosts, striking similarity of effects on the CFU-S and CFU-C in the present tumor-host system may suggest a common effect on all stem cells at various levels of differentiation. The significance of an elevation in the number of various stem cell classes within the spleen of tumor-bearing host is a matter of conjecture. This may fulfil the increased needs of a variety of functional cells: for example, there is an increase in the number of (i) newly-formed lymphocytes within the tumor site [1, 2] and the spleen [6], (ii) newly-formed monocyte-macrophage class of cells within the tumor [1, 2], (iii) granulocytes within the blood and the tumor at later intervals [6], (iv) erythroid and monocyte-macrophage class of cells in the spleen at later intervals of tumor growth [6]. However, the very early increase in the splenic stem cell numbers, which precedes all these phenomena, cannot be regarded as a kinetic consequence of an increased demand for functional cells. Similarly, this event is too early to be explained as effects of possible bacterial endotoxins released from the gut of tumor-bearing hosts. It appears to be a direct effect of tumor development, possibly mediated by antigenic and/or other products of tumor cells. We found that inoculation of tumor cell plasma membranes can produce a rapid increase in the splenic CFU-S content [6]. Various other antigenic materials including bacteria [21] or viruses [22–27] are known to produce similar effects on the distribution of hemopoietic stem cells, although their temporal patterns are somewhat different with different agents.

Tumor transplantation was found to cause a rapid increase in the incidence as well as the total number of splenic lymphocytes capable of producing B lymphocyte colonies in the presence of 2-mercaptoethanol. Macrophages, known to stimulate B lymphocyte colony formation [28] did not show an increase in their numbers within the spleen during the first 5 days of present tumor growth [6] and thus could not account for the above findings. Although the plating efficiency of our assay system was poorer compared to that reported by Metcalf *et al.* [7], present observations on the B lymphocyte colonies are significant in the light of our previous findings on the life history [5, 6] and surface markers [4] of splenic small lymphocytes in the same tumor-bearing hosts. Tumor transplantation caused an increase in the incidence of newly-formed, marrow-derived small lymphocytes within the spleen [5, 6] but no significant changes in the incidence of IgM-bearing B cells recognizable radio-autographically by their binding of ^{125}I-labeled anti-IgM [4]. On the other hand, there was a marked increase in the incidence of "double negative" small lymphocytes which had no detectable surface IgM or *0* antigen [4]; their incidence reached a maximum at 3 days after tumor transplantation followed by a plateau. This temporal pattern was very similar to that shown by the B lymphocyte colony forming cells in the present study. These sets of findings may be reconciled in 2 ways: (i) IgM-bearing B cells in the spleen of tumor transplanted animals had a higher colony forming ability because of a prior *in vivo* stimulation by some products released by the tumor, or (ii) the "double negative" small lymphocytes possessed the potentials to develop into B lymphocytes *in vitro*. Observations of B lymphocyte colony stimulating ability in sera from patients bearing some malignancies [29] may support the first possibility. On the other hand, B lymphocyte colony forming cells may represent a heterogeneous population and the relationship between the degree of differentiation and colony forming ability is unknown [28]. Furthermore, some "double negative" small lymphocytes in the normal mouse bone marrow [30] or human peripheral blood [31] are known to be capable of maturation into IgM-bearing B cells. Such a possibility is very likely also for some of the splenic "double negative" small lymphocytes in the present study. Experiments are in progress to test B lymphocyte colony forming abilities of various lymphocyte subpopulations fractionated from the spleen of tumor transplanted animals. Preliminary results have revealed high B lymphocyte colony forming ability of the "double negative" fraction (Lala and Jacobs, unpublished observations).

In conclusion, present studies combined with

our previous *in vivo* studies [6] on tumor transplanted mice demonstrate a rapid redistribution of pluripotent stem cells, stem cells committed to granulocyte/macrophage development, as well as lymphoid cells capable of producing B cell colonies *in vitro*, possibly initiated by an enhanced migration from the bone marrow to the spleen, where further amplification and differentiation of these cells take place. The precise significance of these responses, although seemingly immunological in nature, remains to be determined.

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Growth and Metastasis of the Lewis Lung Carcinoma in Mice Defibrinated with Batroxobin*

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Abstract—*Lewis lung carcinoma (3LL) cells were implanted intramuscularly into $C_{57}B16J$ mice, rendered hypofibrinogenemic by daily intraperitoneal treatment with batroxobin (17.5 NIH u/kg body wt, twice per day). When this treatment was continued till spontaneous death of the animals, an increase in both metastasis number and weight was observed, but the primary tumor growth was unchanged. In contrast, both metastasis number and weight tended to decrease when defibrination was started from* day 11 *after tumor implantation, independently from surgical removal of the primary tumor.*

These data suggest that fibrinogen and/or its derivatives could play a different role in the various stages of the growth and dissemination of the same experimental tumor.

INTRODUCTION

IN ORDER to gather information on the possible role of fibrin in the processes of tumor growth and metastasis formation, we have previously studied the changes in hemostatic parameters occurring during development of a spontaneously metastasizing tumor, the Lewis lung carcinoma (3LL) [1]. Tumor-bearing animals were found to have higher blood fibrinogen levels, with a reduced plasma half-life of this protein and accumulation of radioactive fibrinogen both in the tumor and in the metastasis-bearing lungs. It was therefore felt of interest to study the growth and metastasis of this tumor in animals maintained on a very low level of circulating fibrinogen by intermittent treatment with batroxobin, a thrombin-like enzyme extracted from the venom of Botrops Moojeni, which converts fibrinogen to fibrin without activating platelets or other clotting factors [2, 3].

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MATERIAL AND METHODS

Animals and tumor

The 3LL tumor was maintained and transplanted intramuscularly in $C_{57}B16$ mice as previously described [1, 4]. In some experiments, the tumor-bearing leg was amputated at day 11 after tumor implantation and the development of pulmonary metastases followed as in non-amputated animals. Metastasis number was evaluated visually by surface count; metastasis weight was estimated by the formula $4/3\ \pi\ r^3$ where r is the semiaxis of each nodule measured on a millimetric scale; the volume was then converted to weight assuming unit density.

Tumor and metastasis growth were measured at spontaneous death of the animals in some experiments; in other experiments, randomly allocated pairs of one control and one batroxobin-treated animal were followed and when either component of the pair died, the counterpart was also killed. These experimental conditions were chosen in order to rule out any differences in the mean survival time between the treated and control group which could interfere in the evaluation of the tumor and metastasis growth.

To study the growth curve of tumor and metastases in control and defibrinated animals,

groups of mice were killed at various intervals starting from day 14. A total of 300 mice was used for this study.

Blood studies

Blood fibrinogen, blood platelet and red cell counts were determined as previously described [1]. Blood was collected from non-anesthetized animals from the retroorbital plexus by means of Pedersen micropipettes and appropriately diluted for the various tests immediately thereafter.

Drugs

Batroxobin (Defibrase®) was kindly supplied by Pentapharm (Basel, Switzerland). Various drug dosages were preliminarly tested to achieve optimal defibrination of the mice. As indicated in Fig. 1, following an i.p. administration of 17.5 NIH units batroxobin per kg body wt,

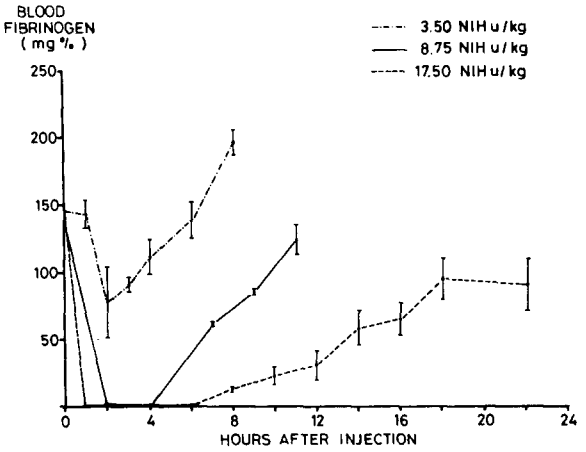

Fig. 1. Blood fibrinogen levels following a single i.p. injection of various dosages of batroxobin into normal mice. For each point, means ± S. E. of values obtained from 5 animals are indicated. The fibrinogen values lower than 8 mg% (sensitivity of the method used) have been arbitrarily reported on the abscissa at zero level. Fibrinogen levels in blood from control animals ranged between 158 and 230 mg%.

the fibrinogen level was lower than 40 mg% during 12 hr; a 12-hr intermittent administration schedule was therefore employed to maintain adequate hypo-fibrinogenemia throughout the experimental period (approximately one month). Batroxobin treatment was started 2 hr before either tumor implantation or amputation (at day 11) and continued till the animals died or were killed. During each experiment, groups of 5–10 animals were used for monitoring the level of defibrination achieved. At 2–3 day intervals, the fibrinogen level was measured immediately before the second daily administration of batroxobin; in 78% of the samples tested, the fibrinogen level was lower than 40 mg%. When the results obtained during the first 15 days of treatment were compared with those obtained in the second half of the observation period, the frequency of fibrinogen values lower than 40 mg% was not significantly different ($\chi^2 = 0.142$). This suggests that tachyphylaxis did not occur.

Control groups consisted of 3LL-bearing mice treated twice daily with i.p. injections of isotonic saline.

Statistical evaluation of the results was performed using Student's t-test or the χ^2 test.

RESULTS

In 3LL-bearing animals defibrinated since the day of tumor implantation, platelet and red cell counts dropped progressively as in control tumor-bearing mice (Fig. 2). The primary tumor weight was not modified by the treatment, whereas both the number and the weight of the pulmonary metastases were increased (Table 1 and Fig. 3).

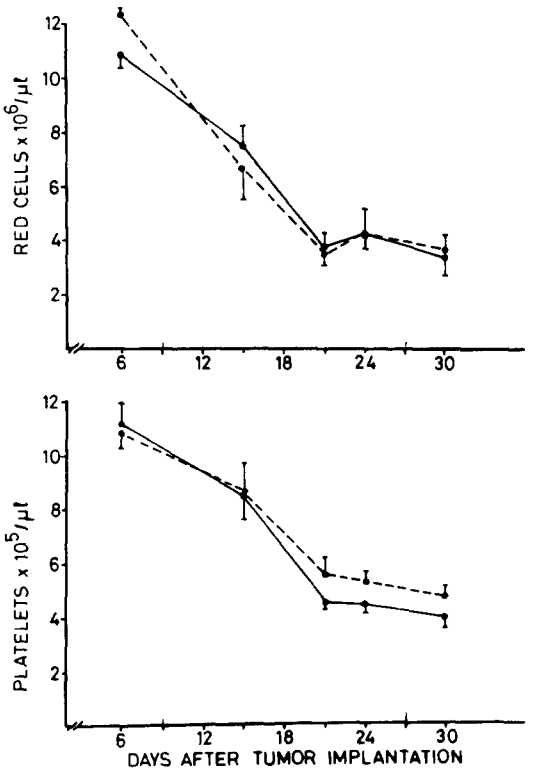

Fig. 2. Evolution of platelet and red cell count in control and batroxobin-treated (broken line) mice during development of the 3LL tumor. For each point, means and S. E. of the values obtained from 5 animals are indicated.

When treatment with batroxobin was associated with surgical removal of the primary tumor, the incidence of local tumor regrowth was significantly reduced ($P < 0.05$); moreover, in batroxobin-treated animals with recurrence of the primary tumor, this was slightly smaller than in control animals (Table 2). The weight and the number of lung metastases developing

Table 1. Effect of batroxobin treatment (started on the day of tumor implantation) on 3LL tumor and metastasis growth

Treatment	Mean survival (days)	Tumor weight (g)	Lung weight (mg)	Metastases Number	Metastases Weight (mg)
Saline	28.0±1.6	8.0±0.8	366±52	18.2±3.1	120±30
Batroxobin		7.9±0.9	417±104	25.5±3.1	244±55*

Means±S.E. of values obtained from 20 couples of animals are indicated.
*$P<0.05$ by Student's t-test.

Table 2. Effect of batroxobin treatment (starting from day 11 after tumor implantation) on the incidence of proximal recurrence and the weight of regrown tumor in mice amputated at day 11

Treatment	Proximal recurrence N/total	Tumor regrowth (g)
Surgery + saline	18/29	3.9±0.4
Surgery + batroxobin	10/29†	2.4±0.3

†$P<0.05$ by χ^2 test.
Means±S.E. are indicated.

in amputated mice with no recurrence of the primary tumor, were also slightly lower in batroxobin-treated animals (Table 3). Similar results were obtained when the mice were defibrinated starting from day 11, without removal of the primary tumor (Table 4).

DISCUSSION

Enhancement in fibrinogen metabolism was observed during the growth and spontaneous metastasis of the 3LL tumor [1]. To further investigate the possible pathogenetic role of fibrin(ogen) in the development of tumor and metastases in this experimental system, we followed 3LL growth in mice rendered hypofibrinogenemic by adminstration of batroxobin, a thrombin-like enzyme. In these conditions, 3LL-bearing mice developed thrombocytopenia and anemia similarly to untreated animals. This suggests that the drop in platelet count already observed in 3LL animals [4] is unlikely to be due to trapping of platelets within intravascular clots and further supports the hypothesis that a defect in platelet production is involved in its pathogenesis [1]. Similarly, the role of intravascular fibrin deposition in the pathogenesis of the hemolytic anemia observed in 3LL mice [1] could be questioned by this observation.

Neither the growth curve nor the weight at death of the primary tumor was modified in defibrinated mice. Similar negative results have been recently reported by De Wÿs *et al.* [5] in 3LL-bearing mice defibrinated by another thrombin-like enzyme, ancrod. However, the lack of effect of thrombin-like enzymes on the primary tumor does not rule out the possibility that fibrin may play some role in this experimental system, especially in relation

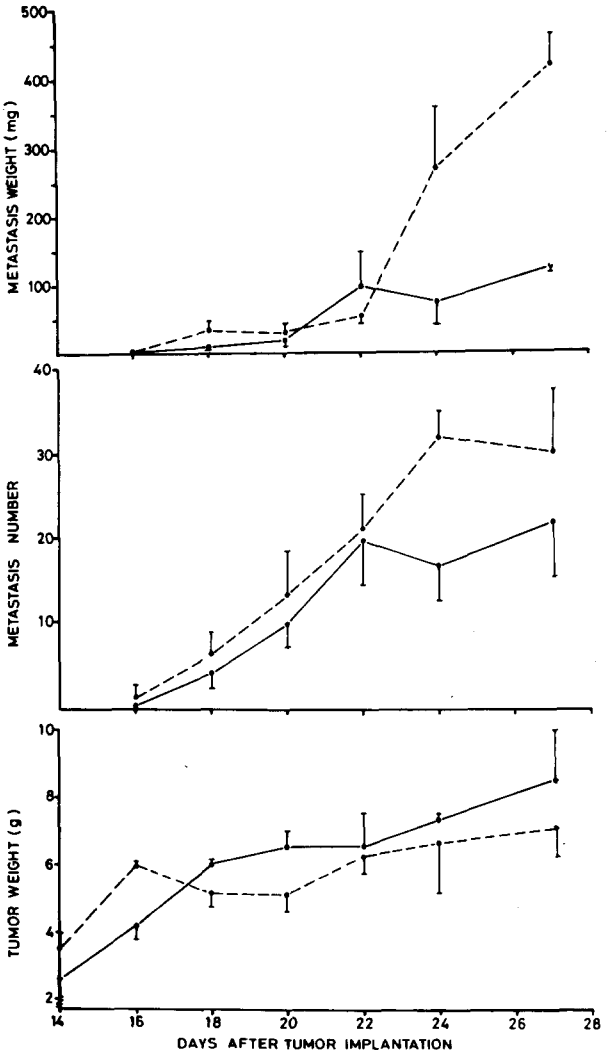

Fig. 3. Growth curves of primary tumor and lung metastases in control and batroxobin-treated (broken lines) mice. For each point, means and S.E. of the values obtained from at least 5 animals are indicated.

Table 3. Effect of batroxobin treatment (starting from day 11 after tumor implantation) on 3LL metastasis growth in amputated mice

Treatment	Mean survival time (days)	Lung weight (mg)	Lung metastases Number	Lung metastases (mg)
Surgery + Saline	24±0.7	455±62	10.4±2.1	150±63
Surgery + batroxobin		353±71	8.2±4.1	101±65

Means±S.E. of values obtained from 10 couples of animals are indicated.

Table 4. Effect of batroxobin treatment (started on day 12 after tumor implantation) on 3LL tumor and metastasis growth

Treatment	Mean survival time (days)	Tumor weight (g)	Lung weight (mg)	Metastases Number	Metastases Weight (mg)
Saline	23.0±1.0	6.9±0.8	383±54	15.6±5.7	115±76
Batroxobin		5.8±0.9	244±19	11.3±3.3	50±31

Means±S.E. of values obtained from 20 couples of animals are indicated.

to the mechanism of metastasis formation. Indeed, the oral anticoagulant warfarin has a more marked inhibitory effect on metastasis formation than on primary 3LL-tumor growth [6, 7]. For this reason, we checked carefully for any possible effect of batroxobin on metastases.

Metastasis formation was enhanced when the mice were defibrinated during the whole tumor development period, whereas it appeared to decrease in mice defibrinated from the time of removal of the primary tumor. To assess whether this opposite trend could be related to the presence or absence of the primary tumor or to the different periods of defibrination, a group of mice was given batroxobin from day 11, but the primary tumor was not removed; in these animals too a tendency to decreased metastasis formation was noted. These observations suggest that fibrin(ogen) might play different roles in the various phases of metastatic spreading of the same tumor. Fibrin might possibly slow the release of cells from the primary mass but favor, as a guideline, their arrest and growth in the lungs.

These results on metastasis formation in batroxobin-treated mice are not easily comparable with data so far available in the literature [8–10].

In previous studies the effects of defibrinating enzymes have been followed applying short-term treatments to artificial "metastasis" systems. Tumor-cells were injected *intravenously* in animals defibrinated at most for 48 hr [8] and the development of either lung or extrapulmonary colonies (rather than metastases) was followed. The possibility of drawing conclusions from these systems on the role of fibrin in metastatic growth therefore seems questionable [1, 6].

Moreover, the studies mentioned were performed on different animal species (mouse, rabbit, rat); differences among the various species in sensitivity to defibrinating enzymes might explain some of the discrepancies between the results obtained with ancrod by Wood and Hilgard [9] in the rabbit and Hagmar [8] in mice. As a more general remark, it is also worth considering that removal of fibrin from the circulation by means of defibrinating enzymes may not necessarily result in altered deposition and/or clearing of fibrin from the tumor or from the metastatic foci; studies on the accumulation of fibrin within the tumor and metastases of batroxobin-treated animals could further elucidate the mechanism of tumor-cell fibrin interaction in the various phases of metastatic development.

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Effects of 3′-Deoxy-3′-fluorothymidine on Proliferation Kinetics of Ehrlich Ascites Tumour Cells *In Vitro* (Comparison with Other Cytostatic Drugs)

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Abstract—*Treatment of Ehrlich ascites carcinoma cells* in vitro *with cytosine-arabinoside, 5-fluoro-2′-deoxyuridine and hydroxyurea in concentrations just completely inhibiting cell proliferation, results, in agreement with data published for other cells, in progressive loss of viability if the treatment exceeds a period of one generation time (about* 14 hr). *Pulse cytophotometric analysis of the treated cells showed them to be located mainly in the G_1-phase of the cell cycle. In contrast, cell proliferation can be inhibited for about* 32 hr *(more than 2 generation times), without loss of viability, if the treatment is done with 3′-deoxy-3′-fluorothymidine (3′-FTdR). Pulse cytophotometric analysis revealed a concentration-dependent delay of progression through the S-phase. At a concentration just completely inhibiting cell proliferation, discontinuation of proliferation was predominant in late S-phase at the G_2-boundary. These properties recommend 3′-FTdR for experiments aimed at improving cancer chemotherapy by a non-toxic accumulation of cells in the S-phase followed by treatment with a second, S-phase specific agent.*

INTRODUCTION

3′-Deoxy-3′-fluorothymidine (3′-FTdR; Fig. 1) is a selective inhibitor of DNA synthesis [1] with cytostatic [2] and virostatic [3] properties. It is

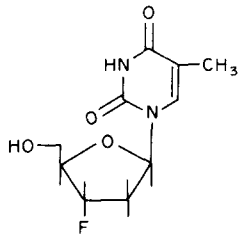

Fig. 1. Structure of 3′-FTdR.

fairly well phosphorylated by thymidine kinase; the resulting 5′-monophosphate is, though poorly, further phosphorylated to the 5′-triphosphate and, finally, incorporated into DNA [4]. Incorporation of 3′-FTdR into DNA will inevitably stop further chain growth, because there is no free 3′-hydorxyl group for the attachment of additional nucleotides.

In the following investigation we show that it is possible with 3′-FTdR to stop proliferation for more than two generations without killing the cells. Moreover, various aspects of inhibition by 3′-FTdR are analysed by pulse cytophotometry (PC).

MATERIAL AND METHODS

3′-FTdR was prepared by the method introduced by Kowollik *et al.* [5]; cytosine-arabinoside (CA), 5-fluoro-2′-deoxyuridine (FUdR) and hydroxyurea (HU) were of commercial origin. Ehrlich ascites tumour (EAT) cells obtained from AB mice about 5 days after transplantation were cultured in suspension as described by Negelein *et al.* [6], except that the sample volume was only 3 ml. The generation time of exponentially growing cells is about 14 hr. Due to a lag phase after transferring the cells to *in vitro* conditions there is only a 2–2.5-fold increase in the cell number during the first

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24 hr. However, results comparable to those described below were obtained with cells which were allowed to proliferate for 16 hr before use. In order to study all effects at comparable concentrations of the inhibitors, we generally used concentrations inhibiting cell proliferation between 80 and 100%. This means that the number of cells in the inhibited samples remained constant or even increased slightly. Such an increase is to be expected, since at the beginning of cultivation about 20% of the cells are always in the G_2-phase and are refractory to inhibition. Though it cannot be excluded that the balance in the cell number is a result of some cell deaths on one hand and some cell divisions on the other hand, we certainly have no major uncontrolled cell losses. The viability of the cells after treatment was determined from their ability to resume growth after removal of the inhibitors and further incubation (usually for 24 hr). During this incubation the medium contained compounds known to counteract the effects of the respective inhibitors (i.e. 2'-deoxycytidine for CA, thymidine for FUdR and a mixture of all four 2'-deoxyribonucleosides for HU; concentration of each nucleoside = 10 μM). Cells which divided during this time were considered to be viable (the surviving fraction). Though this method may not be as sensitive as the usual method of colony counting, its results seem to be comparable (at least for the purpose described in this paper) for the following reasons: (1) the loss of viability determined by this method for the treatment by CA, FUdR and HU is in agreement with published data [7–10] and (2) in all the cases where it was checked, prolonging the incubation for recovery did not alter the degree of recovery.

Cells were counted by an automatic counter TuR ZG2 from then "VEB Transformatoren- und Röntgenwerk Dresden". For PC analysis, cells were fixed in alcohol, treated with RNase and stained with ethidium bromide as described in [11].

For the quantitative evaluation of the histogramms the method of Barlogie *et al.* [12] was used.

RESULTS

1. *Effects on cell proliferation*

Figure 2 shows the effect of the cancerostatic agents investigated on cell proliferation during 24 hr. 3'-FTdR is less effective than CA but more so than HU. On the basis of these results the following concentrations were applied to obtain an 80–100% inhibition of cell proliferation (see Material and Methods): FUdR = 0.002 μM, CA = 1 μM, 3'-FTdR = 10 μM, HU = 200 μM.

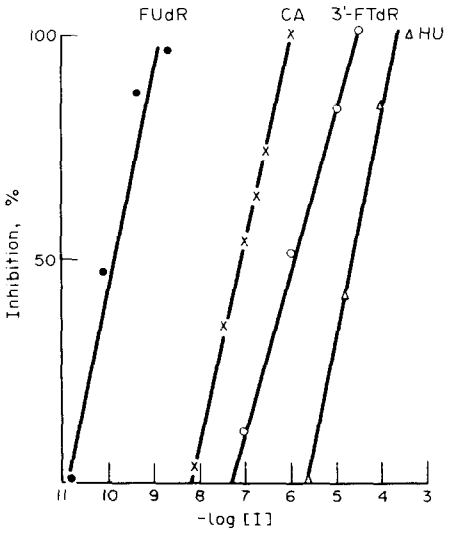

Fig. 2. Inhibition of cell proliferation as dependent on drug concentration. Concentrations are given at the abscissa in negative logarithms of molarity.

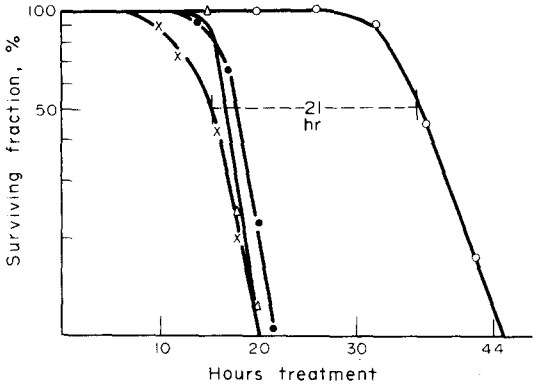

Fig. 3. Effect of drug concentrations inhibiting cell proliferation by 80–100% on cell survival as dependent on treatment time. ×——× 1 μM CA, ●——● 0.002 μM FUdR, △——△ 200 μM HU, ○——○ 10 μM 3'-FTdR.

Figure 3 demonstrates to what extent cells exposed to these inhibitory concentrations for various times recover after removal of the compounds. After treatment for about one generation with CA, FUdR and HU, a strong decrease of the surviving fraction was found. This is in agreement with data published in the literature. Therefore, despite the possible limitations for drawing conclusions from an outgrow assay under the described conditions, we consider the decrease of the growth potential to reflect the cell killing. In contrast, cells can be exposed to 3'-FTdR for a significantly longer period without affecting their growth potential. At higher concentrations, all inhibitors kill the cells more rapidly. With 3'-FTdR, concentrations 5 or 25-fold the dose mentioned above kills about 33 or 81% of the cells within 24 hr,

while in such concentrations the other inhibitors already cause a strong decrease in the cell number (cell loss by lysis). Thus, these results again demonstrate the lower cell killing potential of 3'-FTdR. Lowering the CA, FUdR or HU concentrations resulted in a decrease of both cell killing and inhibition of cell proliferation. It was impossible to find concentrations of these compounds which would inhibit cell proliferation without killing the cells. Approximately 9 hr after the removal of 3'-FTdR there is a burst of mitotic activity, indicating an apparent synchronization, which is lost after one cell division (Fig. 4). However, PC analysis (see below) shows that this is not a "true" synchronous passage through one round of replication, but only a progression from the late S-G_2-phase through mitosis. Thus, there is a rather long phase between removal of the drug and the burst of mitosis (see Discussion).

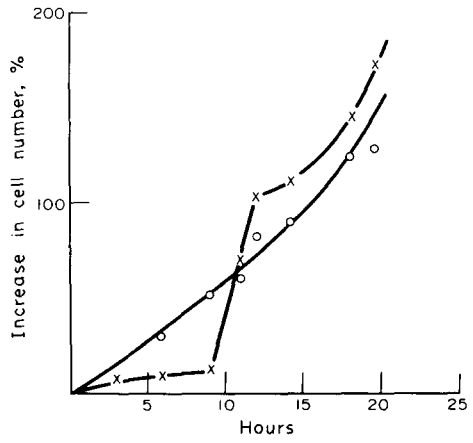

Fig. 4. Time course of cell division after release of a 24 hr *period of 3'-FTdR-inhibition.* O——O *controls,* ×——× *treated cells.*

2. *Effects on progression through the cell cycle*

Figure 5 demonstrates PC analysis of normal cells as well as of cells treated with CA, FUdR, HU or 3'-FTdR in concentrations inhibiting cell proliferation by 80–100%. The controls show the normal distribution of growing cell populations. Usually, with cells of this age we found about 30% cells in G_1, 50% in the S-phase and 20% in the G_2-phase or mitosis. Cells no longer viable after treatment with the killing agents FUdR, CA and HU are found predominantly in the G_1-phase, since there is a strong depletion of S-phase and G_2-phase cells. This is in agreement with recent data on *in vivo* treatment with CA of mice suffering from leukemia L1210 [13].

Quite in contrast, most cells treated with 3'-FTdR (and therefore still viable) are stopped in the late S-phase near the S/G_2-boundary (according to labelling indices, e.g. 84%; they are still mainly in the S-phase, which is also indicated by the PC distribution curves in Fig. 7).

Figure 6 compares the time course of progression through the cell cycle of control and 3'-FTdR treated cells over a period of 24 hr. It follows that progression through the S-phase is strongly delayed by 3'-FTdR (compare the 16 hr

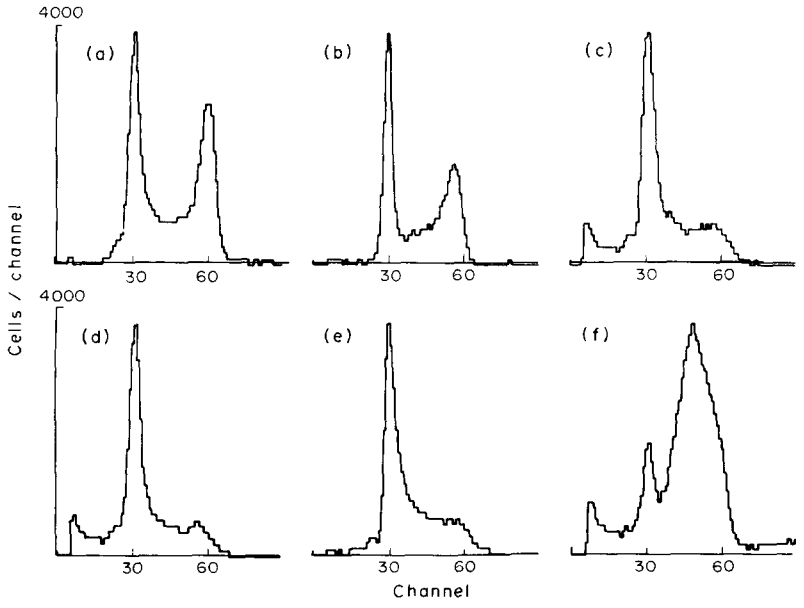

Fig. 5. PC analysis of cells treated for 24 hr *with the same concentrations as in Fig.* 3 *of (C) CA, (D) FUdR, (E) HU and (F) 3'-FTdR. (A) Controls at the beginning of cultivation, (B) controls after* 24 hr *in culture* (132% *increase in cell number).* % *Inhibition: CA* = 95, *HU* = 93, *FUdR* = 97, *3'-FTdR* = 91. *In this and the following figures some of the 3'-FTdR treated samples exhibit a minor peak in front of the* G_1*-peak. This peak consists of cell debris. However, such debris were not always seen and their amount is not correlated to the 3'-FTdR concentration.*

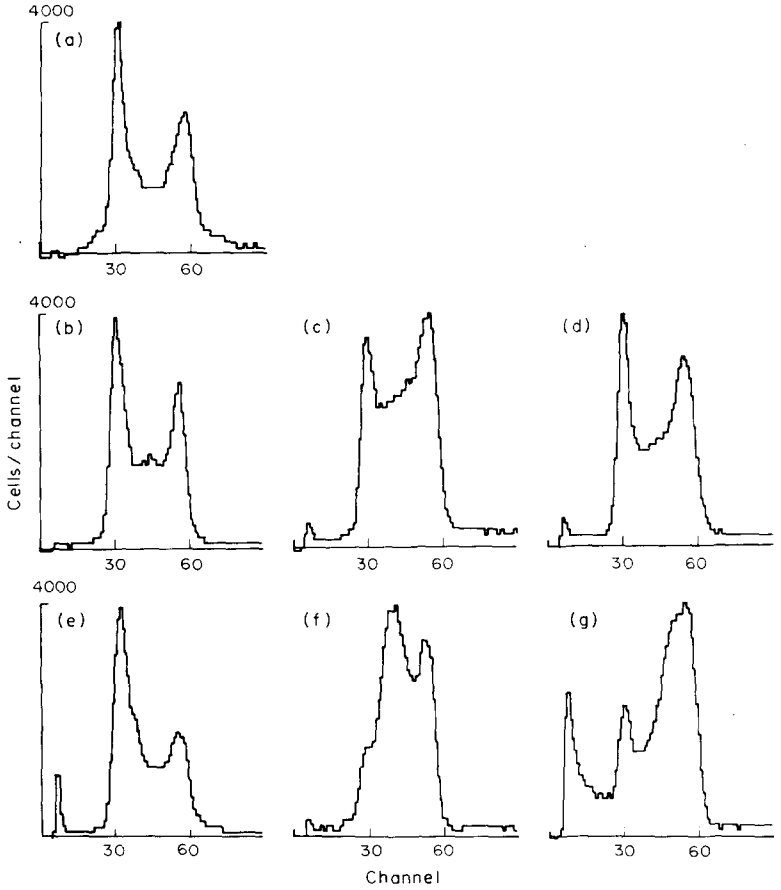

Fig. 6. Effect of 3′-FTdR (same concentration as in Fig. 3) on the progression of cells through the cell cycle over 24 hr. *A, B, C, D = controls at* 0, 8, 16 *and* 24 hr; *E, F, G = treated cells at* 8, 16 *and* 24 hr, 83% *inhibition after* 24 hr *of treatment.*

samples). The same is observed from Fig. 7 showing PC analysis of cells treated with various concentrations of 3′-FTdR (lower or higher than the 80–100% inhibitory concentration). Here with growing concentrations an increasing delay of cell progression through the S-phase is observed, the main cell peak being shifted from the late S-phase at lower concentrations to mid- or early S-phase at higher concentrations.

DISCUSSION

The presented data show that (a) cell killing with 3′-FTdR occurs at a much later time than with the other drugs studied and (b) that 3′-FTdR stops cell proliferation in the (late) S-phase (rather than in the G_1-phase as the other agents). To explain these differences, the respective modes of action have to be considered: FUdR and HU inhibit DNA synthesis by precursor starvation, CA inhibits (as its 5′-triphosphate) DNA polymerases and is incorporated into DNA (also into internal positions of the chain) [14–19]. With either of these compounds inhibition of DNA synthesis seems to be so stringent that further passage through the S-phase is completely prevented. PC analysis showed that in contrast to the other agents 3′-FTdR still permits the cells to pass very slowly through the main part of the S-phase. This effect may considerably delay the loss of viability. As to the mode of action of 3′-FTdR, experiments with intact cells indicated an incorporation of the compound into DNA [1] which was unambiguously demonstrated for cell-free conditions [4]. Since 3′-FTdR inhibition is reversible, the cell must be able to excise the compound or to bypass the blocked pieces. Excision may be facilitated by the terminal position of the analogue. Indeed, a 3′ to 5′-exonuclease activity has recently been demonstrated for a eukaryotic DNA polymerase [20]. As to bypassing, the results of Byars and Kidson [21] should be mentioned; they found that 2′, 3′-didexoythymidine (a very similar nucleoside, differing from 3′-FTdR only in a hydrogen instead of fluorine at the C-3′ position) is incorporated at the end of the growing DNA chain in mammalian cells. The incorporated nucleoside proved to remain in the DNA for many generations of unimpaired cell growth. However, the situation is not fully comparable to that seen with 3′-FTdR,

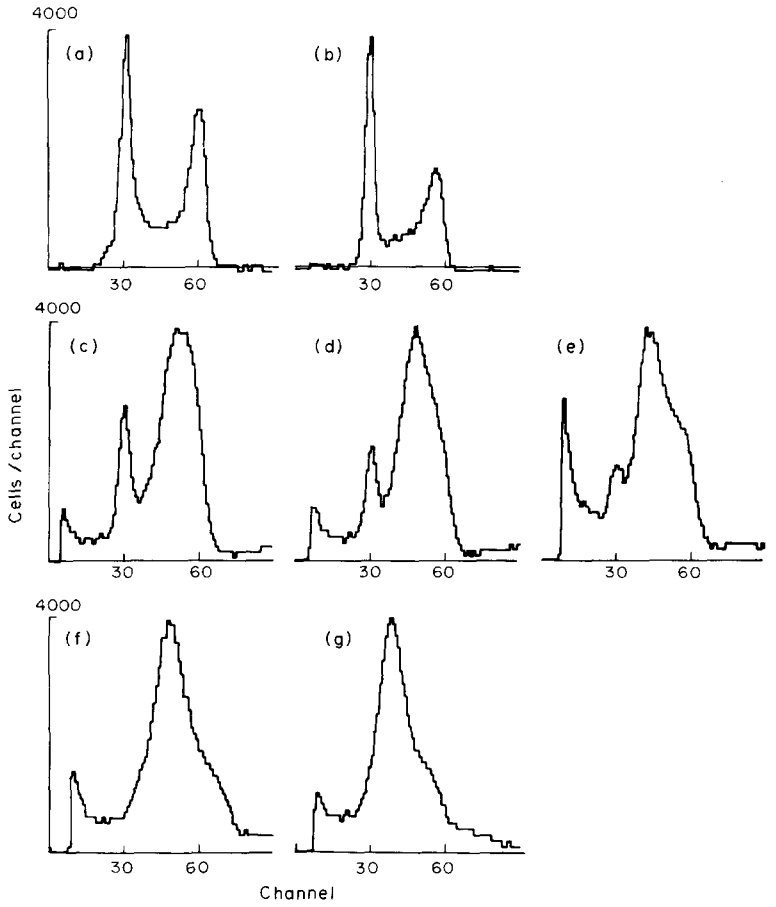

Fig. 7. PC analysis of cells treated for 24 hr *by various concentrations of 3'-FTdR. (A) controls at the beginning of the culture, (B) controls after* 24 hr *in culture* (106% *increase in cell number). Molarity of 3'-FTdR and* % *inhibition: (C)* 3×10^{-6}, 72, *(D)* 1×10^{-5} *(concentration used in Figs.* 3–6), 91, *(E)* 3×10^{-5}, 100, *(F)* 1×10^{-4}, 100 *(G)* 3×10^{-4}, 100.

because—at least in our EAT system—2', 3'-deoxythymidine, even in high doses, has negligible low cytostatic effects.

Wobus [22] found, also in EAT cells, that all of the few cells which undergo mitosis immediately after 3'-FTdR treatment show chromosomal aberrations. In contrast, cells entering mitosis 24–48 hr after removal of the drug showed less or even no aberrations. These results suggest that the lag phase between removal of the drug and the burst of mitosis observed in our experiments may be necessary for repairing at least the most severe lesions which otherwise would give rise to chromosomal aberrations. Preliminary results show that also under *in vivo* conditions (using mice) a dose-dependent arrest of EAT cells and leukemic cells in the late S-phase can be obtained after 24 hr of 3'-FTdR infusion. These results recommend 3'-FTdR as a most suitable agent for the nontoxic accumulation of cells in the S-phase. Usually, the range in cell cycle times is a serious obstacle for a successful chemotherapy with S-phase specific agents like CA [23]. Pretreatment with 3'-FTdR could considerably improve the action of such agents by shortening the necessary period of application. On the other hand, 3'-FTdR may also be used to limit tumour outgrowth during intervals of treatment with highly cytostatic agents.

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The Antileukemic Effect of Systemic Non-specific BCG-immunostimulation vs Systemic Specific Immunostimulation with Irradiated Isogeneic Leukemic Cells*

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Abstract—*The antitumoral effect of BCG has been compared to the antitumoral effect of injections of irradiated isogeneic leukemic cells in a transplantable, murine ascites leukemia. It was found that irradiated tumor cells in doses of* 10^7 *or* 10^9 *cells per injection (either s.c. or i.p.) induced prolongation of mean survival time and increased the percentage of cured mice.*

The effect of irradiated cells was highest with s.c. injections and an effect was observed only in mice inoculated with 10^2 *or* 10^3 *living leukemic cells. BCG had only beneficial effect when given i.v. and only in a dose of* 7×10^6 *viable units per injection. Lower doses of BCG had no effect compared to controls and higher doses given i.v. or i.p. enhanced tumor growth. BCG injected i.v. may induce a cure in mice inoculated with* 10^2–10^5 *living leukemic cells.*

Spontaneously cured mice were highly susceptible to a second tumor inoculum containing a tumor cell number only one decade higher than the first inoculum. In contrast, immunostimulated mice rejected inocula 2–3 decades above first inoculum.

Histologic examination of organs from mice cured either spontaneously or by immunostimulation revealed that BCG induced markedly histiocytic proliferation and infiltration in spleen, thymus, lymph nodes, liver, and lungs, whereas otherwise cured mice had no such lesions.

INTRODUCTION

IT IS well established that administration of immunoadjuvants may lead to tumor growth control in tumor-bearing mice [1, 2]. The adjuvants are classified as specific [3] when they stimulate immunological mechanisms that are supposed to be directed only against the actual tumor and classified as non-specific when they stimulate other activities of the immune system as well [3]. Bacille-Calmette-Guérin (BCG) has proven to be among the strongest non-specific immuno-adjuvants [4, 5], whereas it is more uncertain which specific immunoadjuvant is the most potent.

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Most of these experiments have been performed with transplantable animals tumors, which are often rather strongly antigenic [6] and thus different from spontaneous human tumors that in many cases have shown no evidence of bearing specific tumor associated antigens. This obviously weakens the importance of the experimental results, especially as emphasis is often on immunologic mechanisms that have a neoantigen as prerequisite [7].

We have therfore used a transplantable murine leukemia that in its morphologic [8, 9], immunologic [9] and cytokinetic [8] properties are close to human lymphoid leukemia [10].

A systematic investigation into the effect of non-specific vs specific immunotherapy has not previously been performed in such a model.

In this paper we have concentrated on specific stimulation with isogenic irradiated tumor cells as compared to non-specific BCG-stimulation. We have studied the importance

of the route of administration, dose–response relationship, tumor load and susceptibility to a second inoculum after being cured of leukemia caused by a primary inoculum. Finally, the results of histologic examinations of organs from different groups of cured mice were compared.

MATERIAL AND METHODS

Mice and tumor

Female mice of an inbred $C_{57}Bl/6$ strain were used at 9–10 weeks of age. The mice were inoculated i.p. with various doses of leukemic cells from a transplantable leukemia that has been induced in the $C_{57}Bl/6$ strain by repeated injections of AKR leukemic cells. The tumor is isogeneic with the $C_{57}Bl/6$ mouse and its cytology, growth and various immunological characteristics have been described elsewhere [8, 9]. The tumor is maintained as an ascitic tumor by weekly i.p. injections of 5×10^6 tumor cells into healthy mice. Six-day-old tumor cells were used for inoculation in the various experiments. In each experiment, the mice were randomized into groups of 20 mice that were examined daily, the moribunds killed, and their ascites, liver, cor, kidney, lungs, lymph nodes, thymus, spleen and adrenals examined histologically.

The mice that were cured either spontaneously or by the various treatments were followed 90 days or more and then studied for their ability to reject a second inoculum of tumor cells. Each experimental group contained at least 5 mice. The mice were observed for another 90 days before it was stated whether the second tumor was rejected or not.

Adjuvants

Living BCG was produced at the Pasteur Institute (Paris, France), obtained from this source and comprised $7–10 \times 10^6$ viable units per mg. BCG was dosed according to the number of viable units and injected each week with start at day +1 after tumor cell inoculation. The injections were given either subcutaneously (s.c.), intravenously (i.v.) or intraperitoneally (i.p.) up to 8 times.

Tumor cells for irradiation were harvested from six-day-old ascites tumors. Normal lymphoid cells were obtained from mesenteric lymph nodes from healthy $C_{57}Bl/6$ mice. The cells were exposed to 10,000 rad from a ^{137}Cs-gamma source at a rate of 300 rad/min. The cells were injected immediately after irradiation either i.v., s.c. or i.p., with start at day +1 and given up to 8 times.

Statistics

Wilcoxon's non-parametric test has been used throughout.

RESULTS

I.v. adjuvant administration

Table 1 shows that 5–15% of non-treated mice reject tumor inocula on $10^2–10^3$ cells. 1.4×10^6 viable units of BCG did not alter the survival compared to controls, whereas 7×10^6 units of BCG induced an increase in the number of cured mice when the tumor inoculum was in the order of $10^2–10^5$ cells. The curative effect increases with decreasing number of inoculated tumor cells. In contrast, 21×10^6 units BCG decreased the mean survival time in the groups with $10^2–10^3$ tumor cell inoculum. No cured mice were observed in these groups.

Irradiated normal lymphoid cells as well as irradiated tumor cells had no effect on leukemic cell proliferation when given i.v.

I.p. adjuvant administration

BCG in a dose of 1.4×10^6 units had no effect on tumor growth (Table 3), 7×10^6 and 21×10^6 units of BCG are seen to shorten mean survival time in the 10^2 and 10^3 groups and no cured mice were observed in these groups. Furthermore, no effect of BCG was seen in the other groups with higher tumor cell inocula.

Irradiated tumor cells in a dose of 10^7 or 10^9 cells induced prolongation of mean survival time and increased the number of cured mice (Table 4). No difference was observed between the groups receiving 10^7 irradiated tumor cells or 10^9 cells. Irradiated normal lymphoid cells had no influence on tumor growth.

S.c. adjuvant administration

BCG had no effect on tumor growth (Table 5).

Equally, 10^5 irradiated tumor cells had no effect on tumor growth, in contrast to injections of both 10^7 and 10^9 irradiated tumor cells that highly significantly increased the mean survival time and incidence of cure of mice inoculated with either 10^2 or 10^3 tumor cells (Table 6). In fact, with these inocula the effect of irradiated tumor cells given s.c. is significantly ($P<0.01$) more beneficial than BCG given i.v. However, at 10^5-inoculum no effect was observed with

Table 1. The anti-tumoral effect of various doses of BCG injected i.v. every week with start at day + 1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice >90 days after tumor-inoculation (%)	Statistics*
None (controls)	10^2	26.8±2.3 (19–32)	15	—
	10^3	23.1±1.9 (18–32)	5	—
	10^5	16.5±2.0 (12–20)	0	—
	10^6	15.3±1.6 (12–18)	0	—
1.4×10^6 viable units BCG i.v. per week start day +1	10^2	25.1±2.9 (18–31)	20	N.S.
	10^3	24.0±1.8 (19–30)	5	N.S.
	10^5	15.6±2.1 (11–19)	0	N.S.
	10^6	14.9±1.7 (11–18)	0	N.S.
7×10^6 viable units BCG i.v. per week start day +1	10^2	32.3±4.7 (26–41)	45	$P<0.01$
	10^3	29.3±3.6 (24–36)	35	$P<0.01$
	10^5	17.8+1.9 (13–21)	25	$P<0.01$
	10^6	14.9±2.0 (11–19)	0	N.S.
21×10^6 viable units BCG i.v. per week start day +1	10^2	22.5+1.3 (17–25)	0	$P<0.01$
	10^3	20.3±1.8 (15–24)	0	$P<0.01$
	10^5	14.1±2.0 (11–18)	0	N.S.
	10^6	14.2±2.3 (10–18)	0	N.S.

*Statistics have been performed with Wilcoxons' non-parametric test comparing the survival of all mice in the control group with the survival of the various immune-stimulated groups.

Table 2. The anti-tumoral effect of various doses of irradiated tumor-cells injected i.v. with start at day + 1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice >90 days after tumor-inoculation (%)	Statistics*
None	10^2	24.7±2.3 (17–31)	10	—
	10^3	22.2±2.0 (17–29)	5	—
	10^5	15.6±1.8 (12–20)	0	—
	10^6	14.5±1.8 (12–18)	0	—
10^5 irradiated tumor-cells injected i.v. per week start day +1	10^2	25.2±1.6 (20–29)	15	N.S.
	10^3	23.8±1.9 (18–30)	5	N.S.
	10^5	16.2±2.0 (13–21)	0	N.S.
	10^6	15.5±2.2 (12–20)	0	N.S.
10^7 irradiated tumor-cells injected i.v. per week start day +1	10^2	25.8±2.0 (19–30)	10	N.S.
	10^3	23.0±1.8 (17–29)	0	N.S.
	10^5	15.1±2.1 (11–20)	0	N.S.
	10^6	15.3±2.3 (11–21)	0	N.S.
10^9 irradiated tumor-cells injected i.v. per week start day +1	10^2	28.4±2.2 (24–35)	25	N.S.
	10^3	25.5±2.5 (21–32)	0	N.S.
	10^5	16.0±1.8 (12–21)	0	N.S.
	10^6	14.8±2.0 (11–20)	0	N.S.
10^9 irradiated normal lymphoid cells injected i.v. per week start day +1	10^2	24.0±2.8 (16–31)	15	N.S.
	10^3	23.0±2.2 (16–28)	5	N.S.
	10^5	17.1±2.0 (13–21)	0	N.S.
	10^6	14.3±1.6 (11–20)	0	N.S.

†Statistics have been performed with Wilcoxons' non-parametric test comparing the survival of all mice in the control group with survival of the various immune-stimulated groups.

Table 3. The anti-tumoral effect of various doses of BCG injected i.p. every week with start at day +1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice > 90 days after tumor-inoculation (%)	Statistics*
None (controls)	10^2	25.8±2.1 (20–30)	10	—
	10^3	22.9±2.0 (18–29)	5	—
	10^5	15.6±1.6 (12–20)	0	—
	10^6	14.8±1.4 (11–17)	0	—
1.4×10^6 viable units BCG i.p. per week start day +1	10^2	22.9±2.6 (19–31)	0	N.S.
	10^3	21.2±2.4 (17–28)	0	N.S.
	10^5	15.8±2.0 (12–19)	0	N.S.
	10^6	13.5±1.8 (11–18)	0	N.S.
7×10^6 viable units BCG i.p. per week start day +1	10^2	21.5±1.1 (21–25)	0	$P<0.01$
	10^3	19.2±1.2 (17–23)	0	$P<0.01$
	10^5	14.2±1.1 (12–16)	0	N.S.
	10^6	13.0±1.5 (10–16)	0	N.S.
21×10^6 viable units BCG i.p. per week start day −1	10^2	20.0±2.2 (17–26)	0	$P<0.01$
	10^3	20.4±1.8 (17–24)	0	$P<0.01$
	10^5	15.6±1.6 (13–20)	0	N.S.
	10^6	14.8±2.0 (11–16)	0	N.S.

*Statistics have been performed with Wilcoxons' non-parametric test comparing survival of all control mice with survival of the various immune-stimulated groups.

Table 4. The anti-tumoral effect of various doses of irradiated tumor-cells injected i.p. every week with start at day +1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice > 90 days after tumor-inoculation (%)	Statistics†
None (controls)	10^2	28.0±1.6 (21–33)	15	—
	10^3	23.8±2.0 (19–29)	0	—
	10^5	16.0±1.6 (12–19)	0	—
	10^6	14.8±1.2 (12–17)	0	—
10^5 irradiated tumor-cells injected i.p. per week start day +1	10^2	29.8±3.0 (22–39)	0	N.S.
	10^3	27.2±2.8 (20–34)	0	N.S.
	10^5	15.7±2.1 (12–21)	0	N.S.
	10^6	15.0±1.9 (11–19)	0	N.S.
10^7 irradiated tumor-cells injected i.p. per week start day +1	10^2	40.8±4.0 (28–56)	30	$P<0.01$
	10^3	36.0±2.1 (31–42)	25	$P<0.01$
	10^5	16.6±1.7 (12–19)	0	N.S.
	10^6	15.5±1.4 (11–19)	0	N.S.
10^9 irradiated tumor-cells injected i.p. per week start day +1	10^2	41.0±3.1 (31–52)	25	$P<0.01$
	10^3	36.8±3.0 (30–48)	25	$P<0.01$
	10^5	15.9±1.8 (12–19)	0	N.S.
	10^6	15.4±1.0 (14–17)	0	N.S.
10^9 irradiated normal lymphoid cell injected i.p. per week start day +1	10^2	24.8±2.2 (17–29)	10	N.S.
	10^3	23.1±1.9 (17–29)	0	N.S.
	10^5	15.2±2.0 (11–21)	0	N.S.
	10^6	14.6±2.3 (11–20)	0	N.S.

†Statistics have been performed with Wilcoxons' non-parametric test comparing survival of all control mice with survival of the various immune-stimulated groups.

Table 5. The anti-tumoral effect of various doses of BCG injected s.c. every week with start at day +1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice > 90 days after tumor-inoculation (%)	Statistics*
None (controls)	10^2	24.0±2.2 (19–29)	15	—
	10^3	22.0±1.2 (19–24)	10	—
	10^5	14.7±2.0 (12–19)	0	—
	10^6	14.0±1.8 (11–18)	0	—
1.4×10^6 viable units BCG s.c. per week start day +1	10^2	25.6±1.7 (19–30)	10	N.S.
	10^3	24.4±1.9 (20–30)	10	N.S.
	10^5	16.0±2.2 (11–22)	0	N.S.
	10^6	15.2±1.4 (12–17)	0	N.S.
7×10^6 viable units BCG s.c. per week start day +1	10^2	25.3±2.0 (21–30)	5	N.S.
	10^3	24.1±1.7 (20–29)	5	N.S.
	10^5	15.7±2.0 (12–18)	0	N.S.
	10^6	14.6±1.5 (11–17)	0	N.S.
21×10^6 viable units BCG s.c. per week start day +1	10^2	24.0±2.3 (19–30)	10	N.S.
	10^3	22.8±1.1 (20–26)	5	N.S.
	10^5	16.1±2.0 (11–20)	0	N.S.
	10^6	14.6±1.9 (10–18)	0	N.S.

*Statistics have been performed with Wilcoxons' non-parametric test comparing survival of all control mice with survival of the various immune-stimulated groups.

Table 6. The anti-tumoral effect of doses of irradiated tumor-cells injected s.c. every week with start at day +1 *after tumor-cell inoculation and given maximally 8 times*

Treatment	No. of tumor-cells inoculated	Mean survival time of dying mice in days (range)	Non-tumor bearing mice > 90 days after tumor-inoculation (%)	Statistics†
None (controls)	10^2	28.0±1.8 (21–34)	10	—
	10^3	25.5±2.0 (20–29)	5	—
	10^5	16.8±1.6 (13–19)	0	—
	10^6	14.7±1.4 (12–16)	0	—
10^5 irradiated tumor-cells injected s.c. per week start day +1	10^2	35.2±3.8 (27–42)	10	N.S.
	10^3	32.0±3.6 (28–37)	5	N.S.
	10^5	15.8±2.0 (12–20)	0	N.S.
	10^6	15.0±1.6 (12–19)	0	N.S.
10^7 irradiated tumor-cells injected s.c. per week start day +1	10^2	47.4±6.5 (36–61)	70	$P<0.01$
	10^3	42.7±5.6 (33–54)	45	$P<0.01$
	10^5	17.8±1.4 (14–22)	0	N.S.
	10^6	15.6±2.0 (11–20)	0	N.S.
10^9 irradiated tumor-cells injected s.c. per week start day +1	10^2	43.1±7.0 (34–62)	65	$P<0.01$
	10^3	46.4±4.1 (37–55)	50	$P<0.01$
	10^5	16.3±1.9 (13–22)	0	N.S.
	10^6	14.6±1.1 (12–17)	0	N.S.
10^9 irradiated normal lymphoid cells injected s.c. per week start day +1	10^2	25.0±2.5 (16–31)	10	N.S.
	10^3	22.6±2.0 (17–28)	0	N.S.
	10^5	15.3±1.7 (13–21)	0	N.S.
	10^6	14.3±2.2 (12–21)	0	N.S.

†Statistics have been performed with Wilcoxons' non-parametric test comparing survival of all control mice with the survival of the various immune-stimulated groups.

irradiated tumor cells. Irradiated normal cells had no effect on tumor growth.

Rejection of a second inoculum

Cured mice from repeated experiments of each group were randomized into subgroups of at least 5 mice that were inoculated with tenfold dilutions of a tumor cell suspension, starting one decade higher than the first inoculum.

Almost all spontaneously cured mice died of an inoculum only one decade higher than first inoculum and all mice in this group died with inocula two decades higher than the first inoculum.

Irradiated tumor cells administered i.p. induced resistance to a second inoculum that was 1–2 decades higher than the first one.

Mice cured by irradiated cells given s.c. had a complete resistance to tumor inocula even up to 10^5 tumor cells.

BCG did not induce the same high curability as irradiated tumor cells in the 10^2 and 10^3 groups, but mice cured by BCG however rejected a second inoculum to the same extent as mice cured by irradiated cells (Table 7).

Histologically, all dying mice revealed infiltration in parenchymal tissue. The natural structure of the organs was erased, some degree

Table 7. Rejection ability to a second tumor-cell inoculum i.p. in mice that have rejected a primary tumor-cell inoculum, either spontaneously or induced by injection of BCG or irradiated cells

Treatment after first inoculum*	No. of cells in first inoculum	Non-tumor bearing mice >90 days after tumor-inoculation (%)	No. of cells in second inoculum	No. of takes Total no. of animals
None (controls)	10^2	12	10^3	13/15
			10^4	15/15
	10^3	6	10^4	6/6
10^7 irradiated tumor-cells i.p. per week	10^2	30	10^3	0/7
			10^4	4/7
			10^5	7/7
	10^3	25	10^4	0/9
			10^5	8/8
10^9 irradiated tumor-cells i.p. per week	10^2	25	10^3	0/7
			10^4	7/7
	10^3	25	10^4	1/7
			10^5	6/6
10^7 irradiated tumor-cells s.c. per week	10^2	75	10^3	0/9
			10^4	0/9
			10^5	5/9
			10^6	9/9
	10^3	50	10^4	0/9
			10^5	4/9
			10^6	9/9
10^9 irradiated tumor-cells s.c. per week	10^2	70	10^3	0/8
			10^4	0/9
			10^5	4/9
			10^6	9/9
	10^3	46	10^4	0/7
			10^5	5/8
			10^6	8/8
7×10^6 viable units BCG i.v. per week	10^2	46	10^3	0/6
			10^4	0/6
			10^5	5/6
			10^6	5/5
	10^3	40	10^4	0/7
			10^5	3/7
			10^6	6/6
	10^5	24	10^5	0/6
			10^6	6/6

*See Material and Methods and Tables 1–6.

of histiocytic proliferation was present, but there were no signs of severe BCGosis.

The spontaneously cured mice and mice with irradiated tumor cells revealed no histological lesions at autopsy. In contrast, mice cured by BCG showed histiocytic granulomas in the sinusoids and portal tracts of the liver, in spleen follicles and in peripheral lymph nodes. Infiltrates of histiocytes were also found around the small vessels in the lungs and around arterioli and paraglomerularily in the kidneys. Small histiocytic infiltrates were found between the medulla and cortex of some adrenals and among the myocardial fibrils. Additional findings were cells with abundant PAS positive cytoplasm in the perifollicular zone of the spleen and thymus alterations consisting of marked cortical atrophy and presence in the medulla of some cysts containing a PAS positive, mostly homogeneous material.

DISCUSSION

The present paper confirms and extends previous studies on the antitumoral effect of specific (irradiated tumor cells) vs non-specific (BCG) immunostimulation [11–14].

The effect of BCG and irradiated cells is highly dose-dependent [15, 16]. The present work permits a direct comparison between the effect of BCG and irradiated cells, showing that BCG is more efficient than irradiated cells with increasing tumor-inoculum, the reverse being true with decreasing tumor load. BCG could even enhance tumor growth, whereas such an effect was not observed in any case during treatment with irradiated tumor cells.

The benign histological reactions may be due solely to BCG, as non-tumor-bearing mice can show the same lesions after BCG-treatment.

The work of many groups has amply demonstrated that immunologic mechanisms may be cytotoxic to tumor cells *in vitro*. As argued previously, many of these results may not be relevant to human neoplasms due to differences in antigenicity, but even in appropriate models little evidence exists to support the hypothesis that the immunological mechanisms induced by immunoadjuvants play any important role in whatever tumor-regression is observed [17].

This implies that we consider both immunologic and non-immunologic mechanisms as possible candidates for the antitumoral effect [18].

In contrast to BCG, irradiated tumor cells in no case decreased the survival time or cure-expectancy. The best results were obtained with subcutaneous administration. The results were even superior to those induced by BCG in mice with small tumor inocula (10^2 or 10^3), whereas BCG was superior to irradiated cells in mice receiving large inocula (10^5).

The histiocytic reaction observed in mice cured after i.v. BCG treatment is in accordance with that described by Rappaport and Khalil [19], as is also the case with the cyst-like of the thymus from BCG treated animals. Such cysts containing PAS positive fluid may occur spontaneously in very old mice and may be induced in large numbers by estrogen treatment [20, 21]. The biological importance of this PAS positive fluid is unknown.

The efficiency of immunostimulation in human acute lymphoblastic leukemia is still debated as it has been postulated that the frequency of complete remissions is the same in patients receiving chemotherapy only as compared to a protocol combining chemotherapy and stimulation. Recently, it has however been claimed that whereas some patients treated with chemotherapy only, relapse after 3–4 yr in remission, no such late relapses are seen in patients receiving immunostimulation in addition to chemotherapy [1].

We think that our experiment concerning rejection of a second tumor cell inoculum is in favour of this argument for using immunostimulation as mice spontaneously cured were highly susceptible to a second inoculum and immunostimulated mice quite resistant to a second inoculum. The present work thus demonstrates that administration of immunoadjuvants may induce tumor regression and resistance to a second tumor inoculum. The results also suggest that the antitumoral mechanisms by BCG are different from those induced by irradiated cells. Further studies remain to elucidate these mechanisms, which must be known before immunostimulation can be applied optimally to the cancer patient.

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Uptake and Subcellular Localization of Daunorubicin and Adriamycin in Cultured Fibroblasts*

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Abstract—*Cellular uptake and subcellular localization of daunorubicin and adriamycin were studied in cultured rat embryo fibroblasts. Both drugs accumulated in the cells manifold above the extracellular concentration. The uptake of daunorubicin was 2–3 time higher than that of adriamycin. Cell fractionation techniques, including isopycnic centrifugation of cytoplasmic extracts, revealed that both drugs were exclusively localized in nuclei and lysosomes, but to quite different proportions. About 20% of accumulated daunorubicin was localized in the nuclei, as compared to 60% of adriamycin, which means that the nuclear concentrations of the drugs were very similar. In the lysosomes, on the other hand, the accumulation of daunorubicin was 4–5 times higher than that of adriamycin. The reason for this difference is so far not clear.*

The results give an explanation for observations of similar biologic effects of the drugs in spite of a marked difference in cellular accumulation.

INTRODUCTION

ALTHOUGH very similar in their chemical structures, daunorubicin (DNR) and adriamycin (ADR) are characterized by important but poorly understood differences in their pharmacological and therapeutic properties [1]. The antitumoral activity of these drugs is most probably related to their affinity for DNA, characterized by their intercalation between two successive DNA base pairs [2, 3].

The DNA binding properties of DNR and ADR are very similar *in vitro* [4], as could be expected by the evidence that the anthraquinone side chain, by which the drugs differ, does not take part in the intercalation process [3]. The accumulation of DNR has been shown to be consistently higher than ADR in L1210 and Ehrlich ascites cells [5–7]. While in the same cells, their effects on DNA and RNA synthesis are similar and cannot easily be correlated with the levels of intracellular drugs [5, 8].

We have tried to better understand the cellular pharmacology of DNR and ADR by studying their intracellular uptake and localization in cultured cells using the technique of cell fractionation. Our first studies, reported here, were performed on rat embryo fibroblasts, which have been extensively studied and characterized by this technique [9].

MATERIAL AND METHODS

Cell culture

Rat embryo fibroblasts were obtained and cultivated as described by Tulkens *et al.* [9]. For incubation with drugs, cells from the first or second subculture were used as soon as they reached confluency.

Uptake experiments

For the drug uptake experiments, cells were grown in Falcon®-T-flasks (25 cm^2 of growth

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surface, which corresponds to ±1 mg of cell protein). After incubation, cells were washed three times with PBS (phosphate buffered saline, pH 7.4, NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM), harvested in this medium by scraping (with a rubber "policeman") and finally disrupted by ultrasonication (15 sec at 75 W, 20 kHz: Sonifier B-12, Branson Sonic Power Company, Conn. USA).

Cell fractionation procedures

After incubation with drugs, cells grown in Roux flasks (200 cm^2 growth surface) were washed three times with PBS and harvested by scraping after an incubation of 15–30 min with ice-cold EDTA (0.5 mM in PBS). For each fractionation experiment, 5 Roux flasks were used corresponding to ±50 mg of cell proteins. Cells were homogenized, fractionated and enzymes and proteins assayed as described earlier [9]. Cell fractionation involves the preparation of a cytoplasmic extract by differential centrifugation and the centrifugation of the various components of this extract to their equilibrium density in a sucrose gradient. The distribution of the various marker enzymes and of the drugs was plotted in the form of standardized histograms [10].

Drugs assays

ADR and DNR were assayed by fluorometry using excitation and emission wavelengths of 485 and 580 nm, respectively. In order to release the drugs from their association with cellular constituents, 1 ml aliquots of sonicated cell suspension or fractions were treated with 2 ml TCA (40%) and 0.1 ml BSA (10%) as coprecipitant. The drugs were then measured in the supernatant obtained after a low speed centrifugation. The drug concentration in each sample was calculated by comparison with identically treated standard solutions.

Metabolites of DNR and ADR were studied by thin layer chromatography of samples extracted from the cell suspensions with a mixture of chloroform-methanol (2:1). Silica gel plates and two different solvent systems were used: CH_2Cl_2–CH_3OH–HCOOH–H_2O (85:15:2:1) and $CHCl_3$–CH_3OH–H_2O (120:20:1).

Materials

All chemicals were of analytical grade. DNR-hydrochloride was kindly supplied by Rhône-Poulenc (Paris, France) and ADR-hydrochloride by Farmitalia (Milano, Italy).

RESULTS

Drug accumulation

The cellular uptake of DNR and ADR after various times of incubation at a 17.5 μM concentration is given in Fig. 1. The accumulation reaches a steady state level after 10–15 hr for both drugs, while the amount of DNR associated with the cells is about three times higher than that of ADR. The percentage of fluorescent drug recovered in the cells and the incubation medium at various times with respect to the starting fluorescence, is given in Table 1 and indicates that no loss of fluorescence occurs during incubation up to 6 hr. The absence of a major metabolic conversion of ADR or DNR by fibroblasts was confirmed by thin layer chromatography. Only trace amounts of daunorubicinol and adriamycinol were found after 14 hr of incubation.

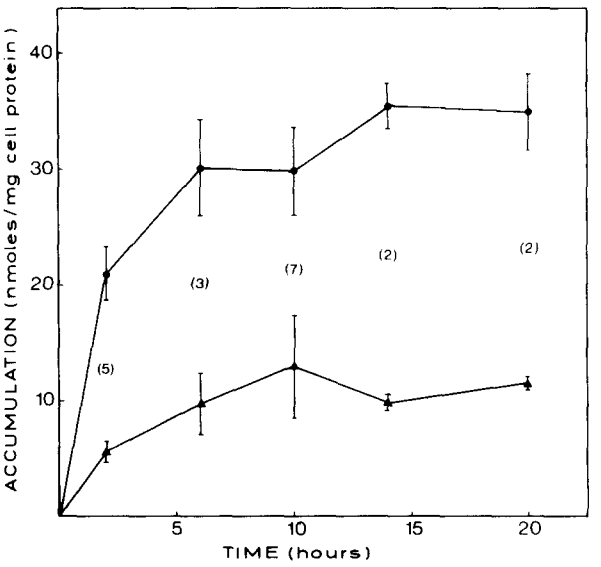

Fig. 1. Accumulation kinetics of DNR (circles) and ADR (triangles) in fibroblasts incubated at an extracellular drug concentration of 17.5 μM *(mean values ± S.D.). Figures within parentheses give numbers of experiments.*

Table 1. Drug fluorescence recovered in fibroblasts and incubation medium after incubation with DNR or ADR (17.5μM) *for different times*

Incubation time (hr)	Daunorubicin (% recovered)	Adriamycin (% recovered)
2	102 ± 3.0	98 ± 3.0
4	97 ± 3.5	97 ± 1.2
6	108 ± 2.3	103 ± 0.6

The table gives the percentage of drug fluorescence found after the incubation as compared to the starting medium (mean values ± S.D. of 3 experiments).

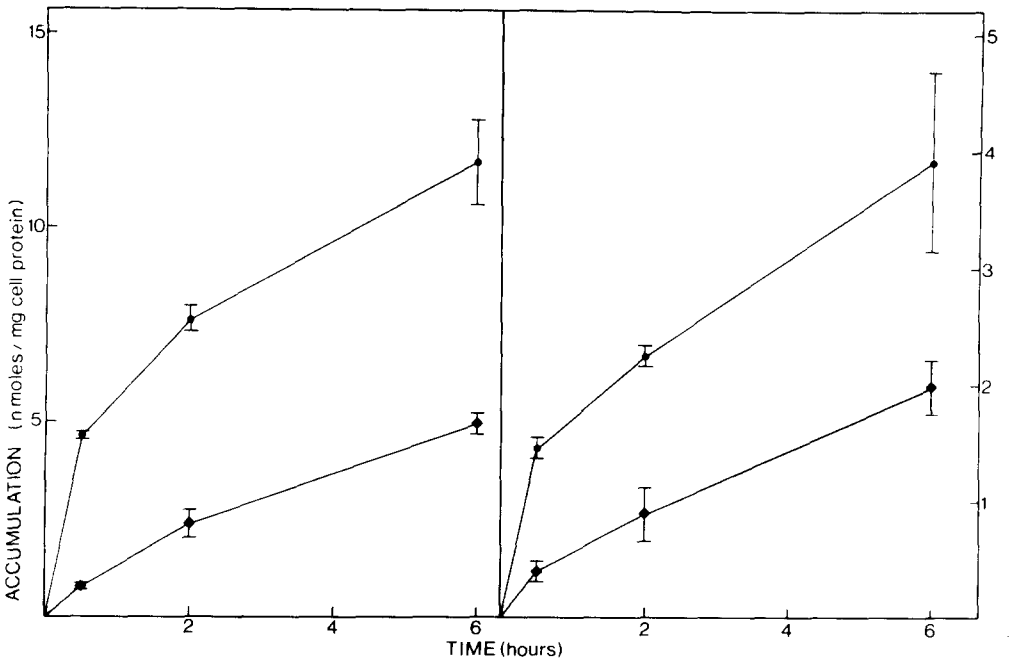

Fig. 2. Accumulation kinetics of DNR (circles) and ADR (squares) in fibroblasts incubated at an extracellular drug concentration of 5.25 μM *(left) and* 1.75 μM *(right). Mean values ± S.D. of 3 experiments are given.*

An about 2 fold difference in accumulation between DNR and ADR is also observed, as shown in Fig. 2, when the cells are incubated with the drugs at lower extracellular concentrations (5.25 μM and 1.75 μM).

In order to investigate whether ADR could affect the uptake of DNR, fibroblasts were incubated with a mixture of DNR and ADR at different proportions with a constant total drug concentration (17.5 μM). As shown in Fig. 3 the total drug uptake during 1 hr increases linearly with the percentage of DNR present in the mixture.

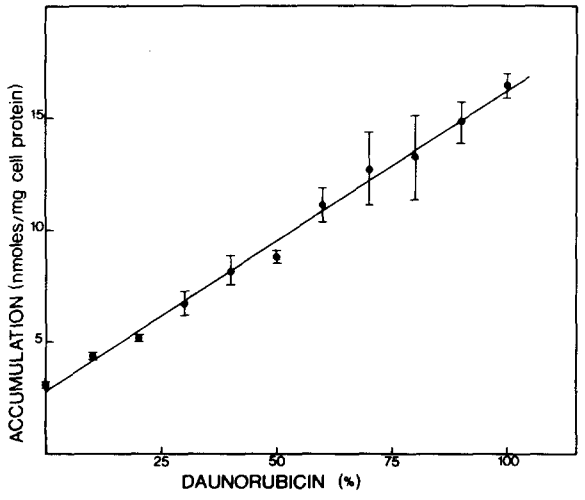

Fig. 3. Accumulation of fluorescent drugs in fibroblasts incubated for 1 hr *with a mixture of DNR and ADR in different proportions at a total concentration of* 17.5 μM. *Accumulation is presented as a function of the percentage of DNR in the mixture (mean values ± S.D. of 3 experiments).*

Subcellular localization of DNR and ADR

After incubation with DNR or ADR (17.5 μM) for 10 hr, fibroblasts were fractionated into a nuclear fraction and a cytoplasmic extract. The absence of nuclei in the cytoplasmic extract was verified by microscopy. The distribution of the drugs and of various marker enzymes in these two fractionations are given in Tables 2 and 3. The localization of DNR and ADR in the cytoplasmic extract was subsequently analyzed by equilibrium density centrifugation.

As shown in Figs. 4 and 5 the distribution patterns of DNR and ADR are clearly distinct from those of cytochrome oxidase (marker enzyme for mitochondria), NADPH: cytochrome c reductase (marker enzyme for endoplasmic reticulum) and 5′-nucleotidase (marker enzyme for plasma membrane). On the other hand, their distributions correspond closely to those of *N*-acetyl-β-glucosaminidase and cathepsin B, suggesting very strongly an association of both drugs with lysosomes.

Control experiments in which DNR was added to a fibroblast homogenate gave completely different results: the distribution of the drug in the cytoplasmic extract overlapped very

Table 2. Distribution of DNR, protein and marker enzymes between cytoplasmic extract and nuclear fraction in fibroblasts incubated with DNR (17.5 μM) for 10 hr

	Total value	Cytoplasmic extract(%)	Nuclear fraction (%)
DNR	40.34 nmole/mg cell protein	69.4	30.6
Protein	48.89 mg	80.0	20.0
Cytochrome oxidase	26.50 mU/mg cell protein	89.6	10.4
NADPH: cytochrome c reductase	7.32 mU/mg cell protein	84.1	15.9
N-acetyl-β-glucosaminidase	25.97 mU/mg cell protein	79.2	20.8
Cathepsin B	7.30 mU/mg cell protein	79.6	20.4
5′-nucleotidase	35.79 mU/mg cell protein	84.2	15.8

Table 3. Distribution of ADR, protein and marker enzymes between cytoplasmic extract and nuclear fraction in fibroblasts incubated with ADR (17.5 μM) for 10 hr

	Total value	Cytoplasmic extract (%)	Nuclear fraction (%)
ADR	6.22 nmole/mg cell protein	41.6	58.4
Protein	49.38 mg	73.7	26.3
Cytochrome oxidase	8.40 mU/mg cell protein	90.5	9.5
NADPH: cytochrome c reductase	4.12 mU/mg cell protein	85.9	14.1
N-acetyl-β-glucosaminidase	21.70 mU/mg cell protein	74.3	25.8
Cathepsin B	4.30 mU/mg cell protein	76.6	23.4
5′-nucleotidase	47.81 mU/mg cell protein	85.4	14.6

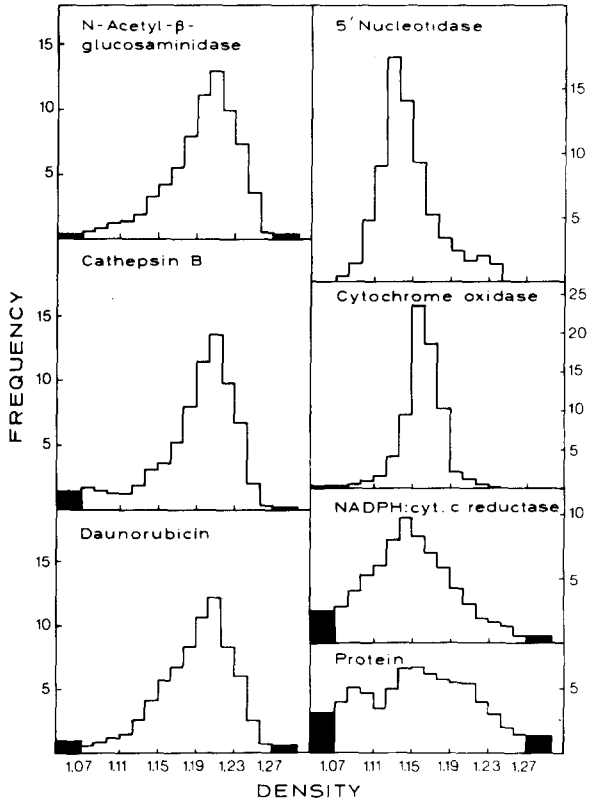

Fig. 4. Distribution of DNR, protein and marker enzymes in the cytoplasmic extract of fibroblasts incubated with the drug for 10 hr at an extracellular concentration of 17.5 μM. The frequency (ordinate) is $\Delta Q/(Q \times \Delta\rho)$, where ΔQ is the amount of constituent in the section. Q the total amount of the constituent in all sections and $\Delta\rho$ the density increment for each section (0.0133). Solid areas represent the amount of constituent recovered at densities below 1.07 and above 1.27.

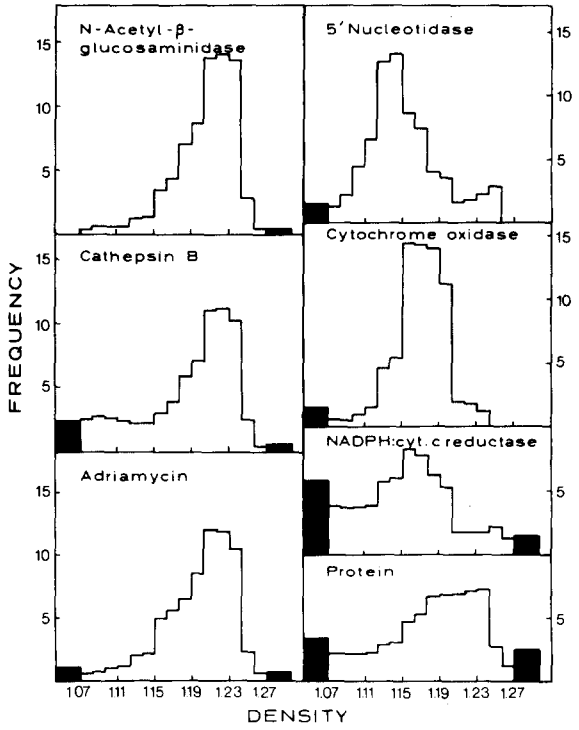

Fig. 5. Distribution of ADR, protein and marker enzymes in the cytoplasmic extract of fibroblasts incubated with the drug for 10 hr at an extracellular concentration of 17.5 μM.

closely the distribution of protein. This indicates that a redistribution of drug during the homogenization process cannot account for the results obtained with homogenates prepared from cells incubated with the drugs.

Since cytoplasmic DNR and ADR are exclusively localized in lysosomes, it becomes possible to estimate the nucleo-cytoplasmic distribution of the drugs on the basis of experiments reported in Tables 2 and 3. However, the nuclear fractions are contaminated with cytoplasmic components and therefore contain lysosomes. Taking into account the relative amount of *N*-acetyl-*β*- glucosaminidase and cathepsin B in the nuclear fraction, one can estimate the true distribution of DNR and ADR between the nuclei and the lysosomes. As illustrated in Fig. 6, these results indicate that for

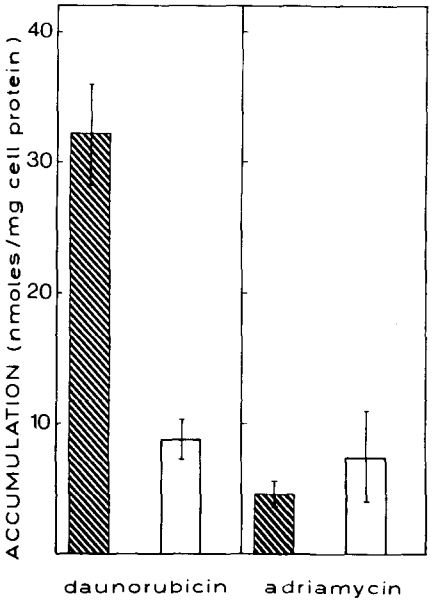

Fig. 6. Distribution of DNR and ADR between nuclei (open bars) and lysosomes (hatched bars) in fibroblasts incubated for 10 hr *with the drugs at an extracellular drug concentration od* 17.5 μM *(mean values ± S.D. of* 4 *experiments).*

the same extracellular concentration of drugs, the nuclear contents of DNR and ADR are very similar. The difference in intracellular concentration between the two drugs is exclusively accounted for by higher lysosomal concentration of DNR.

Similar nucleo-cytoplasmic distributions were obtained when cells were incubated for 8 hr at lower extracellular concentrations (5.25 and 1.75 μM).

DISCUSSION

As described earlier for other cell types [5, 6, 7] DNR and ADR are taken up in fibroblasts to a very high extent. We can evaluate from the data of Fig. 1 that DNR and ADR accumulate in the cells 800 and 150 times, respectively, as compared with the extracellular concentration. These results are obtained if we assume that 1 μg of fibroblast proteins corresponds to a cellular volume of 5 μl [11].

However, intracellular DNR and ADR do not distribute uniformly throughout the cells but accumulate in the nuclei and in the lysosomes. From Fig. 6 and Tables 2 and 3 we can calculate that after 10 hr of incubation at an external drug concentration of 17.5 μM, ±20% of intracellular DNR and ±60% of intracellular ADR are localized in nuclei. The concentrations of drug in the nuclei are very similar, while the lysosomal concentration of DNR is much higher than that of ADR. If we assume that the lysosomes occupy 4% of the total fibroblast volume [12], we can estimate that DNR is concentrated about 20,000 times and ADR about 3750 times inside the lysosomes as compared with the extracellular concentration.

The association of both drugs to nuclei is easy to explain by their affinity for DNA. The lysosomal accumulation can, on the other hand, be explained by the properties of weak bases of DNR and ADR. These drugs could indeed be able to permeate through cell membranes under their non-protonated form and become trapped inside the lysosomes, wherein the lower pH would increase very much the proportion of drugs existing under a protonated form, which would be unable to cross the lysosomal membrane [13]. We cannot however explain, on this basis, the difference in lysosomal accumulation observed between DNR and ADR, since these drugs have very similar pK_a-value [7, 14]. ADR does not interfere with the simultaneous accumulation of DNR since, as shown in Fig. 3, there is a linear relationship between total drug accumulation and the percentage of DNR in the mixture of DNR and ADR present in the incubation medium. This seems to rule out an effect of ADR on the storage capacity of the lysosomes e.g. by an effect on the intralysosomal pH.

An active outward transport for DNR at the plasma membrane level has been claimed by Danø *et al.* [15] and Skovsgaard [7] to exist in Ehrlich ascites cells. The differences in lysosomal accumulation observed between DNR and ADR could be explained by a higher affinity of such a transport system for ADR than for DNR as a consequence a higher extrusion rate for ADR from the cells. Since both drugs show similar nuclear concentrations, we have to postulate that ADR has a 7-fold larger affinity than DNR for nuclei. *In vitro*, R. Baurain (pers. comm.) has observed a significantly larger binding constant of ADR towards DNA, in comparison with DNR.

Our observations can also, if extended to other cell types, explain the higher specific inhibitory effect of ADR on nucleic acid synthesis and cell

growth, as compared to DNR, since a higher proportion of ADR is localized in the nuclei. The results could similarly explain, to some extent, the higher *in vivo* toxicity of ADR. Since after i.v. injections, DNR and ADR are cleared from the blood stream at the same rate [16], a greater proportion of DNR in the tissues should be stored in lysosomes wherein the drug is unable to exert its toxic action.

However, our results obtained with fibroblasts can only be extended to other cell types with great precautions. We have indeed to keep in mind that the amount of drug accumulated inside the lysosomal system of a given cell type will depend on the lysosomal volume of the cells. While the lysosomes occupy more or less 4% of the cellular volume of fibroblasts, other cells may have a smaller lysosomal volume, like for example hepatocytes (0.8%) [17] and rat hepatoma cells (1.9%) (P. Baudhuin, personal communication). On the other hand, the proportion of drug bound to the nuclei could be much higher in tumor cells if these cells are tetraploidal and contain a relatively higher amount of DNA. In fact, Skovsgaard [7] has claimed that less than 10% of DNR and ADR is localized in the lysosomes of Ehrlich ascites cells.

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The Serum Immunoglobulins in Lymphoproliferative Malignancies*

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Abstract—*Immune deficiency may be a prerequisite for or a consequence of the development of malignancies. This seems particularly true for lymphoproliferative disorders in which the proliferation of malignant cells and alterations of immunologic functions are closely connected. Our study was undertaken to determine whether the level of serum immunoglobulins is modified early and during the course of lymphoproliferative disorders. One hundred and thirty-two patients (38 Hodgkin's disease, 57 non-Hodgkin's lymphoma and 37 chronic lymphatic leukemia) were investigated at the time of diagnosis; 48 were reinvestigated after 6 months and 35 after 12 months. When mean values were considered, IgG was found to be higher (though not significantly) than in controls in the first stages of Hodgkin's disease; by contrast IgM was significantly lower from stage II to stage IV. No significant modification was found in lymphomas, while in CLL, IgG, IgA and IgM were definitely lower than in controls. CLL also disclosed the highest frequency of association of lowered immunoglobulins. Immunoglobulin levels did not show significant modifications during the course of the lymphoproliferative disorders studied. The scattering of individual values was the same in the three groups of malignancies and was not closely related to the stage of Hodgkin's disease and lymphoma.*

INTRODUCTION

LYMPHOCYTES belong to three main populations, T, B or null, characterized by different functions and various surface markers. Most non-Hodgkin's lymphomas, including chronic lymphocytic leukemia (CLL), are of B cell origin, while Hodgkin's disease probably results from a disorder of T lymphocytes [1, 2]. CLL and Hodgkin's disease are attended by well described immunologic deficiencies whereas such deficits are less well documented in solid non-Hodgkin's lymphomas [3–6].

The aim of the present study was to re-evaluate the problem of immunoglobulin levels in lymphoproliferative disorders with the prospect; (1) of detecting early isolated or associated modifications that could suggest the pre-existence of an immune deficiency, (2) of determining whether differences of either diagnostic or prognostic value can be established between Hodgkin's disease, CLL and the other non-Hodgkin's lymphomas, (3) of seeing whether significant modifications of immunoglobulin levels can be observed during the course of these disorders.

Although immunoglobulin level in CLL has been the subject of several publications [6–12], this problem has received little attention in the other non-Hodgkin's lymphomas [13] and in Hodgkin's disease [12–14].

Our results show that IgM in Hodgkin's disease, and IgG, IgA and IgM in CLL are significantly lower than in controls, that CLL has the highest rate of association of lowered immunoglobulins and that no significant modifications can be detected during the course of these diseases within a short period (12 months) of follow-up.

MATERIAL AND METHODS

Patients and controls

One hundred and nineteen normal subjects, 60 males and 59 females aged 20–80 yr and uniformly distributed throughout all age classes were used as controls.

One hundred and thirty-two patients (Table 1) who were seen in the Division of Onco-Hematology from 1968 to 1974 were investigated at the time of diagnosis and again after 6 and 12 months for 48 and 35 of

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Table 1. Diagnosis, stage, and histological classification of patients

Diagnosis, sex and age	Stage*	Number of cases	Classification	Number of cases
Hodgkin's disease	I	6	Nodular sclerosis†	22
(19 M, 19 F)	II	11	Mixed cellularity	10
Mean age 37.5 yr	III	16	Lymphocyte predominance	3
(range 15–62)	IV	5	Lymphocyte depletion	3
Total		38		
Non-Hodgkin's lymphomas	I	2	Poorly differentiated lymphocytic lymphoma‡ Centroblastic lymphoma§	17
(25 M, 32 F)	II	21	Nodular lymphocytic lymphoma‡ Centroblastic/centrocytic lymphoma§	14
Mean age 57.7 yr	III	19	Well differentiated lymphocytic lymphoma‡ Centrocytic lymphoma§	19
(range 18–84)	IV	19	Histiocytic lymphoma‡ Immunoblastic lymphoma§	7
Total		57		
Chronic lymphocytic leukemia (18 M, 19 F) Mean age 65.5 yr (range 42–88)				
Total		37		

*Staging according to the Ann Arbor Conference (Carbone *et al.* 1971).
†Classification according to the Rye Conference (Lukes *et al.* 1966).
‡Classification according to Rappaport (1966).
§Classification according to the Kiel Conference (Lennert *et al.* 1975).

them respectively. In all cases of Hodgkin's disease and of solid non-Hodgkin's lymphomas (the latter will be referred to as "lymphomas"), the diagnosis was established on clinical, radiological and histological examination. Patients with CLL were analyzed as a separate group and the diagnosis was based on bone marrow and peripheral blood examination.

Staging was done according to the Ann Arbor Conference [15]. Histological classification was established according to the Rye Conference [16] for Hodgkin's disease and according to Rappaport [17] and Lennert *et al.* [2] for non-Hodgkin's lymphomas.

None of the patients with CLL was treated during the study. All patients with Hodgkin's disease and lymphomas were treated with either radiotherapy or chemotherapy including one or several drugs and corticosteroids following initial determination of serum immunoglobulin levels. It must also be stressed that, except in one case, none of our patients complained of recurrent infections that could evoke an immunodeficiency during the years that preceded the diagnosis of lymphoproliferative disorder.

Determination of serum immunoglobulins

Blood was obtained by venipuncture and sera were stored at −20°C until used. Quantitative estimation of immunoglobulins was performed by radial diffusion in antibody-agar plates using 3 dilutions of each serum. Tripartigen plates (Behringwerke, Marburg, Germany) were used for IgG and IgA, and Immunoplates (Hyland Laboratory, Los Angeles, California) for IgM.

Immunoelectrophoresis

Immunoelectrophoresis was performed according to the micromethod of Scheidegger [18] using horse antiserum to whole human serum (Hyland) and rabbit antisera to IgG, IgA, IgM and kappa and lambda chains from our own stock that were rendered monospecific by appropriate absorptions.

Statistical analysis

The student's *t*-test was used to evaluate the significance of the results.

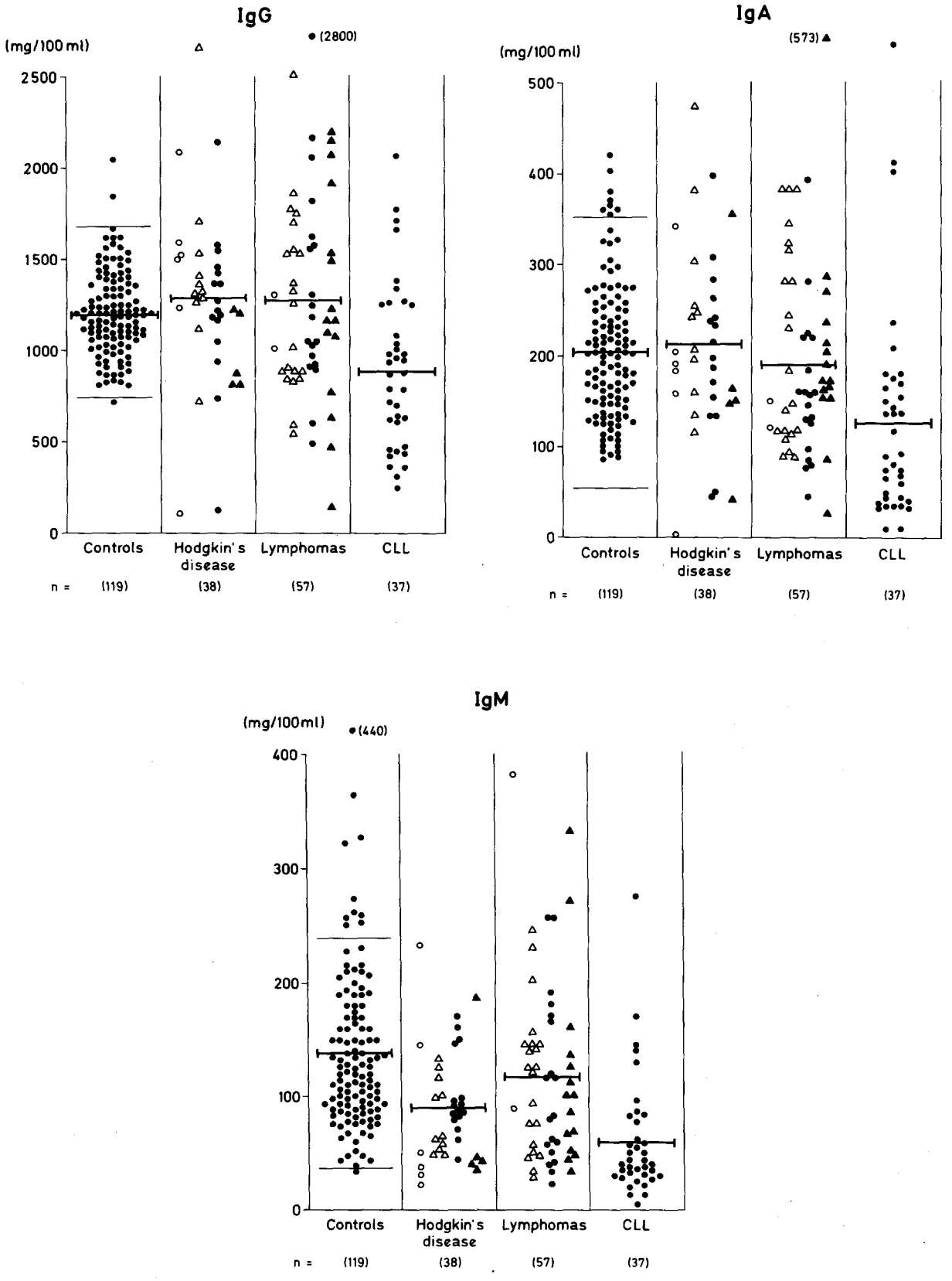

Fig. 1. A, B, C. *Immunoglobulin levels in the serum of patients with lymphoproliferative disorders. Horizontal bars express mean values and, in controls,* ±2 *standard deviations from the mean. For Hodgkin's disease and lymphoma:* ○, *stage* I, △, *stage* II, ●, *stage* III, ▲, *stage* IV.

RESULTS

Immunoglobulin levels in controls and in lymphoproliferative disorders (Fig. 1, Tables 2 and 3)

From the data presented in Fig. 1, it appears that low values were more frequent than high values whatever the type of disorder studied and also that this frequency does not depend upon the stage of Hodgkin's disease or lymphoma. The average values of each group show that the IgG level was slightly higher in Hodgkin's disease and lymphoma than in controls though these differences were not significant. In Hodgkin's disease also, IgM was lower than in controls and in CLL all three immunoglobulins were significantly lower than in all other groups (Fig. 1, Table 2). When the different stages of Hodgkin's disease and of lymphoma were considered, significant differences were found in the former but not in the latter group. IgG was higher than in controls in stages I, II and III of Hodgkin's disease but only in stage II was this difference significant. IgG was lower than normal in stage IV. As for IgM, it was significantly lower than in controls in stages II, III and IV (Fig. 1, Table 3). Three patients, two with Hodgkin's disease and one with lymphoma, were striking for their low IgG level. The first patient had been treated for several years for recurrent respiratory infections before Hodgkin's disease developed. He was known to have very low IgG and IgA and normal IgM. When Hodgkin's disease stage I was diagnosed, immunoglobulin values were as follows: IgG 107, IgA 0 and IgM 185 mg/100 ml. He eventually died from septicemia following splenectomy. The second patient who had Hodgkin's disease stage III had essentially lowered IgG (IgG 123, IgA 133 and IgM 92 mg/100 ml). The third patient had lymphoma stage III and rheumatoid arthritis: immunoglobulin levels were IgG 150, IgA 140 and IgM 235 mg/100 ml.

Association of immunoglobulins with levels higher or lower than normal

Table 4 presents the frequency of abnormally high and low values as well as the frequency with which two or three immunoglobulins with abnormal values were associated in one patient. IgG was found to have elevated values in a relatively small number of cases

Table 2. Significance of differences in immunoglobulin levels between controls and lymphoproliferative disorders

Comparison between groups	IgG	IgA	IgM
Hodgkin/controls	N.S.	N.S.	$P<0.01$
Lymphomas/controls	N.S.	N.S.	N.S.
CLL/controls	$P<0.01$	$P<0.001$	$P<0.001$
Hodgkin/lymphomas	N.S.	N.S.	N.S.
Hodgkin/CLL	$P<0.01$	$P<0.001$	$P<0.01$
Lymphomas/CLL	$P<0.01$	$P<0.01$	$P<0.001$

Table 3. Immunoglobulin levels (in mg/100 ml) in lymphoproliferative disorders according to stage

Diagnosis	Stage	Number of cases	IgG (Mean ± 2 S.D.)	IgA (Mean ± 2 S.D.)	IgM (Mean ± 2 S.D.)
Controls		119	1204.3 ± 468.4	204.0 ± 148.8	138.7 ± 103.2
Hodgkin's disease	I	6	1356.1 ± 1323.4	181.1 ± 228.0	122.0 ± 184.4
	II	11	1405.0 ± 936.0*	242.3 ± 215.6	84.6 ± 77.8*
	III	16	1267.6 ± 908.4	204.8 ± 169.0	93.6 ± 75.4*
	IV	5	985.0 ± 404.6*	165.0 ± 105.2	65.6 ± 75.0*
Lymphomas	I	2§	1165.0 ± 438.4	137.5 ± 43.8	231.0 ± 421.4
	II	21	1292.6 ± 1131.0	155.7 ± 164.8†	105.9 ± 147.8
	III	19	1273.3 ± 1019.6	218.8 ± 209.8	119.1 ± 127.8
	IV	15	1208.6 ± 1132.2	202.3 ± 238.8	109.5 ± 166.2
CLL		37	886.0 ± 883.8†	126.0 ± 228.4†	59.5 ± 106.0†

*†Significantly different from controls, *: $0.05>P>0.01$; †: $P<0.01$.
§t-test not performed for this group.

Table 4. Frequency of immunoglobulins with levels higher or lower than 2 S.D. in controls and in lymphoproliferative disorder

		Controls $n=119$		Hodgkin $n=38$		Lymphomas $n=57$		CLL $n=37$	
		n	%	*n*	%	*n*	%	*n*	%
Ig with levels higher than 2 S.D.	IgG	2	1.6	4	10.5*	13	22.8*	3	8.1
	IgA	8	6.4	4	10.5	5	8.8	3	8.1
	IgM	10	8.0	0	0	6	10.5	1	2.7
Association of Ig with levels higher than 2 S.D.	3Ig	0	0	0	0	1	1.8	0	0
	2Ig	0	0	2	5.2	0	0	1	2.7
	1Ig	20	16.0	4	10.5	21	36.8*	5	13.5
Ig with levels lower than 2 S.D.	IgG	1	0.8	4	10.5*	7	12.3*	16	43.2*
	IgA	0	0	1	2.6	2	3.5	12	32.4*
	IgM	1	0.8	4	10.5*	5	8.8*	15	40.5*
Association of Ig with levels lower than 2 S.D.	3Ig	0	0	0	0	1	1.8	8	21.6*
	2Ig	0	0	1	2.6	1	1.8	5	13.5*
	1Ig	2	1.6	7	18.4*	9	15.8*	9	24.3*

Significance of differences.
*$P<0.05$ when compared to controls.

Table 5. Differences in immunoglobulin levels at time of diagnosis and after 6 and 12 months of evolution in lymphoproliferative disorders

Diagnosis	Number of cases	IgG mg/100ml	*P**	IgA mg/100ml	*P*	IgM mg/100ml	*P*
Hodgkin's disease							
At diagnosis	18	1226.2	—	197.4	—	90.3	—
After 6 months	18	1362.9	N.S.†	171.3	N.S.	84.0	N.S.
After 12 months	12	1050.8	N.S.	151.7	N.S.	78.0	N.S.
Lymphomas							
At diagnosis	23	1386.2	—	197.8	—	108.7	—
After 6 months	23	1364.7	N.S.	190.3	N.S.	118.3	N.S.
After 12 months	18	1119.7	N.S.	158.6	N.S.	140.2	N.S.
CLL							
At diagnosis	11	952.2	—	159.6	—	55.3	—
After 6 months	7	889.1	N.S.	177.2	N.S.	63.8	N.S.
After 12 months	5	1067.0	N.S.	209.2	N.S.	85.2	N.S.

**P*: *t*-test between values at time of diagnosis versus values at 6 and 12 months after diagnosis.
†N.S.: not significant.

of Hodgkin's disease and in a greater number of lymphoma without relationship to the stage of the disease. Only the lymphoma group had a significantly higher frequency of at least one elevated immunoglobulin as compared to controls. By contrast, decreased values were found more frequently. The number of cases with low IgG and IgM was greater in all patients groups than in controls while, for IgA, this was the case only in CLL. The most striking group was CLL since 2 or 3 immunoglobulins were lowered in 13 patients, i.e. 35.1% of cases. It is remarkable that in none of our patients did we find divergent abnormalities with, for instance, the association of one immunoglobulin above and one below 2 standard deviations.

Differences in immunoglobulin levels during the course of disease

Forty-eight patients investigated at the time of diagnosis could be explored after 6 months of evolution and 35 were reinvestigated after 12 months. As shown in Table 5, the average value of the three immunoglobulins tested was not significantly modified in any of the three groups of patients. However, this type of investigation made sense only in the CLL

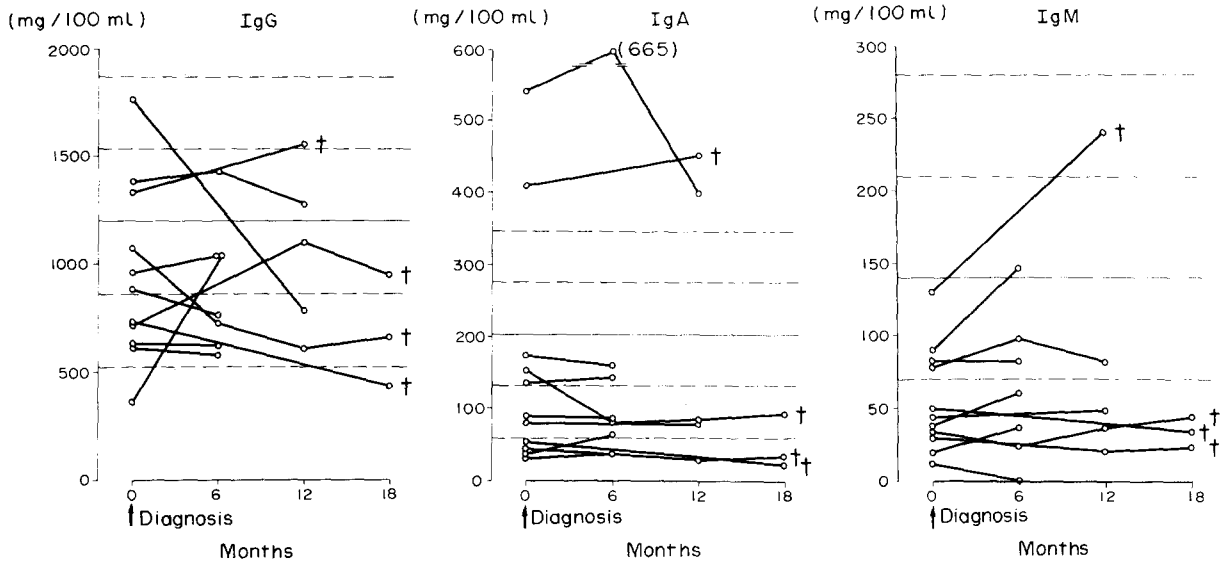

Fig. 2. Immunoglobulin levels during the course of chronic lymphocytic leukemia. —— mean value of 119 controls; --- one and two standard deviations from the mean.

group, since all patients with Hodgkin's disease and lymphoma were treated in the meantime while patients with CLL were not. Eleven of these patients were reinvestigated at least once after 6, 12 or 18 months. As shown in Fig. 2, an increase or decrease greater than one standard deviation was observed on 4 occasions for IgG and one occasion for IgA and IgM. No clear correlation could be established between these modifications and the course of the disease. It is, however, remarkable that among the four patients who died, three had low levels of immunoglobulins, particularly IgA and IgM. One died from heart failure at 73, one from miliary tuberculosis at 73 and one from severe anemia and gastrointestinal hemorrhage at 71. The patient with elevated immunoglobulins had alcoholic cirrhosis and died from hepatic failure at 68.

Presence of M-components

Immunoelectrophoresis did not reveal the presence of M-component in any of the patients investigated.

DISCUSSION

Our data show that the scattering of individual values was approximately the same in all three groups of lymphoproliferative disorders studied; however only in Hodgkin's disease and CLL were mean values significantly different from normal. In Hodgkin's disease, IgG was rather elevated in the first stages and lowered in stage IV, while IgM was constantly lowered from stages II to IV. Elevated IgG has been reported by other authors [12, 14], while some controversy exists as for data regarding IgM. Decreased IgM level has been reported by Waldmann *et al.* [12], while IgM was found mostly within normal limits by Gajl-Peczalska *et al.* [11] and elevated by Wagener *et al.* [14]. The reason for these discrepancies is not clear since our patients, as well as patients reported in the other studies, were not treated at the time of investigation.

Immunoglobulins were, on the average, little affected in lymphomas though individual high and low values were encountered. This is at variance with the observations of Gajl-Peczalska *et al.* [11] who reported low average values for IgM (if one excepts one case of Waldenström macroglobulinemia) and normal values for IgG and IgA. In our cases, no significant differences were noted depending on the stage of the tumor. It was not attempted to establish correlations between immunoglobulin levels and the histological type of lymphoma; these correlations were not evident in another study [11].

CLL was characterized by immunoglobulin levels lower than in controls and other lymphoproliferative disorders, as was already reported [7, 9, 10, 12]. In addition, the CLL group disclosed the most frequent association of lowered immunoglobulins. Although patients were investigated at the time of diagnosis, it could not be decided whether hypogammaglobulinemia was present at the beginning of the disease, since CLL gives usually few

clinical symptoms and is often overlooked in its early stage. However, it is known that the longer the duration of the disease, the more frequent the chances to discover hypogammaglobulinemia [6, 9]. In addition, decreased rate of IgG synthesis has been demonstrated in CLL [19].

The elevation of immunoglobulin levels, particularly IgG, that was observed in some patients, especially in the group of Hodgkin's disease and lymphoma may well represent a humoral response to tumor antigens. These antibodies might be cytotoxic and contribute to the destruction of the malignant cells; alternatively, they might be blocking and enhance the proliferation of the tumor. The hypothesis that patients with high immunoglobulin levels do better than those with normal or low immunoglobulin levels still awaits confirmation. Regarding the first question we asked ourselves before starting this investigation, it is evident that the decrease of one or more immunoglobulin(s), although present in some early stages of Hodgkin's disease and lymphoma, was not frequent enough to support the hypothesis of a pre-existing humoral immune deficiency. Also, the clinical history of our patients was not in favour of this possibility. Though decreased immunoglobulin levels were the most frequent in CLL, it could not be ascertained which disorder, hypogammaglobulinemia or proliferation of malignant cells, came first. The answer to the second question depends upon whether mean values or individual immunoglobulin levels are considered. On a statistical basis, a decrease of two or three immunoglobulins was significantly more frequent in CLL, and a decrease of IgM was more frequent in advanced stages of Hodgkin's disease than in controls and in lymphoma. However, cases with high or low immunoglobulin levels were found in all three groups of lymphoproliferative disorders and in all stages of Hodgkin's disease and of lymphoma. This makes it difficult to retain the determination of immunoglobulin levels as a convenient method of differential diagnosis of the various lymphoproliferative disorders. As for the third question, it could not be shown that immunoglobulin levels were significantly modified during the course of the lymphoproliferative disorders studied and three of the four CLL patients who died had low immunoglobulin levels already at the time of diagnosis. It must be stressed that our follow-up was relatively short for a disease which has usually a prolonged course.

Clearly, lymphoproliferative disorders affect immunologic functions in quite a different way than other malignancies do, although both types of tumors probably result from an initial deficit of immune surveillance or from immune escape mechanisms: severe alterations of immune reactions which may appear in late stages of non-lymphoid malignancies are often present early in lymphoproliferative disorders. It is also remarkable that different types of tumoral processes originating in the lymphoid tissue such as those we have studied modify to various extents such a simple parameter as immunoglobulin levels, even when two of these processes, CLL and solid non-Hodgkin's lymphoma, have in most cases the same B cell origin.

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Melanogenuria in Hamsters with Transplantable Melanoma

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Abstract—*Little has been known about the excretion of melanogens (melanogenuria) in animals with experimental melanoma. In this report the first information about melanogenuria in hamsters with transplantable melanotic and amelanotic melanoma is given. In hamsters with melanotic melanoma apparently increased concentration and excretion of so called Thormählen positive melanogens in urine was found in comparison with hamsters with amelanotic melanoma or hamsters without tumours. Similar results were obtained when the excretion of all diazo positive compounds in urine was measured.*

Thin-layer chromatography of ethyl acetate extracts of hamster urine proved the presence of at least 15 phenolic or indolic acids. Some of them remained unidentified. However, such compounds as p-hydroxyphenylacetic acid, homovanillic acid, vanilmandelic acid or 5-hydroxyindole-acetic acid were identified. Only in the urine of hamsters with melanotic melanoma were we able to detect the isomeric 5-hydroxy-6-methoxyindolyl-2-carboxylic and 6-hydroxy-5-methoxyindolyl-2-carboxylic acids.

INTRODUCTION

THE URINARY excretion of melanogens (melanogenuria) in human subjects suffering from malignant melanoma has been studied for many years [1–8] and two principal groups of melanogens have been distinguished, i.e. Thormählen positive and Thormählen negative melanogens [9]. While there was a lot of knowledge collected, practically nothing was known about melanogenuria in animals with experimental melanomas. Only in 1970 Procházková and Duchoň described the presence of homovanillic and vanillactic acids in the urine of mice with Harding–Passey melanoma [10] and in the same year Käser *et al.* described the presence of the aforesaid acids in the urine of hamsters with transplantable melanoma type V 15 [11]. The isolated reports refer to occurrence of 3,4-dihydroxyphenylalanine in the urine of animals with experimental melanoma [12–14].

In the present paper we want to report on the first information about melanogenuria in hamsters with transplantable melanotic and amelanotic melanoma.

MATERIAL AND METHODS

Animals and tumours

Golden Syrian male hamsters were used in all studies. The tumour source was melanotic and amelanotic melanoma supplied by the courtesy of Dr. Bomirski (Poland). The properties of both related tumour lines have been described by Bomirski and coworkers [15, 16].

Two or three hamsters were placed in each glass metabolic cage (Velaz—Prague) and 24 hr urine collection was carried out. Urine was collected in glass bottles of metabolic cages containing 0.2 ml of 10% $NaHSO_3$. Hamsters without tumours served as controls.

Photometric tests

The concentration of Thormählen positive melanogens was measured in daily urinary samples of hamster with melanotic and amelanotic melanoma and controls using a quantitative modification of the Thormählen reaction [17] as follows:

One fifth to a half of a millilitre of urine was mixed with 1 ml of 0.2% sodium nitroprusside. Two millilitres of 10% KOH was added to this mixture and contents of test tube was well mixed. Finally, the solution was acidified by 0.2 ml of concentrated HCl. The absorbance at 620 nm was determined in 20 min.

The amounts of Thormählen positive melanogens in urine were expressed in terms of mg equivalent of indole, calculated using the standard curve for indole.

The quantity of all phenolic compounds in

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urine able to react with diazotized *p*-nitroaniline was measured according to Gan *et al.* [18].

Ethyl acetate extraction and thin-layer chromatography

The Thormählen negative melanogens were also studied qualitatively. Fifty millilitres of urine were acidified to pH 1 by HCl (indicator paper) and extracted twice with the same volume of ethyl acetate. The pooled extracts were dried with anhydrous sodium sulphate and evaporated to dryness in vacuo. The extract was dissolved in 1 ml of methanol and an aliquot of 20 µl was applied to thin-layer of cellulose (20 × 20 cm, Lucefol—Kavalier, Votice) and subjected to two-dimensional chromatography in the following solvents: I. Isopropanol–ammonia–water (8:1:1), II. anisole–acetic acid–water (69:30:1) [19]. The time of development was 180 min (I.) and 30 min (II.) respectively. The detection was carried out by diazotized *p*-nitroaniline in alkaline medium, u.v. light, Ehrlich's reagent and "ammoniacal silver nitrate".

Statistics

The results were evaluated statistically by Student's *t*-test.

RESULTS

The results of total excretion of Thormählen positive melanogens were expressed either as concentration values (µg equivalents of indole/ml) or as mean excretion values of the quantity of melanogens excreted by 1 hamster for 1 day (µg equivalents of indole/24 hr). Figure 1 illustrates the values of concentration and Fig. 2 the excretion in a group of hamsters with melanotic melanoma, amelanotic melanoma and without tumours. The values are summarized in Tables 1 and 2.

The quantity of all phenolic compounds in urine of hamsters capable of reacting with diazotized *p*-nitroaniline was measured according to Gan *et al.* [18]. The results are given in Fig. 3 and in Table 3.

In all cases the difference between the group of hamsters with melanotic melanoma on the one hand and with amelanotic melanoma or the control group on the other hand were considered significant with the probability of 99%.

The Thormählen negative melanogens were studied by comparing two-dimensional thin-layer chromatograms of ethyl acetate extracts of urines in each group (Fig. 4).

As is shown in Fig. 4 we confirmed the presence of the previously described compound

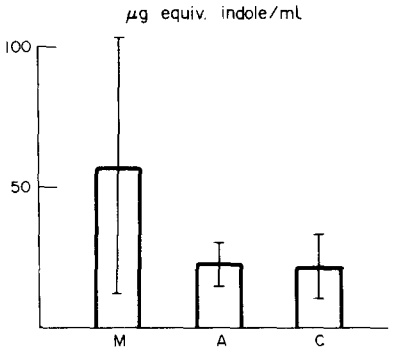

Fig. 1. Concentration of Thormählen positive melanogens in urine of hamsters. M—hamsters with melanotic melanoma; A—hamsters with amelanotic melanoma; C—hamsters without tumors (controls). The values are numerically summarized in Table 1.

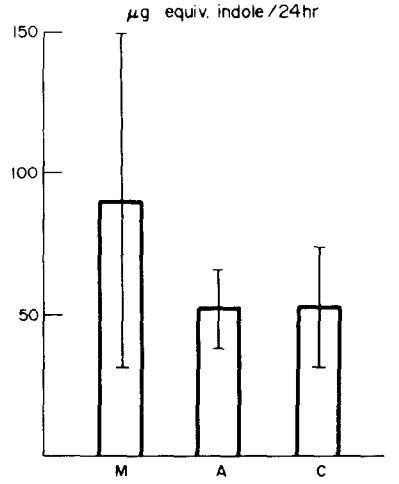

Fig. 2. Excretion of Thormählen positive melanogens in urine of hamsters. M—hamsters with melanotic melanoma; A—hamsters with amelanotic melanoma; C—hamsters without tumours (controls). The values are numerically summarized in Table 2.

—homovanillic acid—in the urine of hamsters with melanotic melanoma. Nevertheless, homovanillic acid was present in the urine of hamsters with amelanotic melanoma and hamsters without tumours, too. We turned our attention to the next Thormählen negative melanogen, whose chromatographic behaviour, and particularly the fluorescence in u.v. light, were strikingly similar to the melanogen described in 1967 by Duchoň and Matouš [3] in the urine of melanoma patients. These authors [3] identified the compound as a mixture of isomeric 5-hydroxy-6-methoxyindolyl-2-carboxylic and 6-hydroxy-5-methoxyindolyl-2-carboxylic acids. We were able to elucidate the identity of this Thormählen negative melanogen as a mixture of the isomeric acids above mentioned (to be published).

DISCUSSION

Figures 1 and 2 illustrate that the excretion

Table 1. Concentration of Thormählen positive melanogens in hamster urine

Experimental groups	No. of hamsters	Concentration (mean ± S.D.) (μg equiv. indole/ml)
Melanotic melanoma	148	57.0 ± 46.0
Amelanotic melanoma	32	22.6 ± 7.8
Controls	62	21.4 ± 10.8

Table 2. Excretion of Thormählen positive melanogens/24 hr

Experimental groups	No. of hamsters	Excretion (mean ± S.D.) (μg equiv. indole/24 hr)
Melanotic melanoma	148	89.7 ± 58.9
Amelanotic melanoma	29	51.5 ± 13.6
Controls	39	52.4 ± 21.2

Table 3. Excretion of diazo positive compounds/24 hr

Experimental groups	No. of hamsters	Excretion (mean ± S.D.) (μg equiv. tyrosine/24hr)
Melanotic melanoma	84	15.21 ± 6.60
Amelanotic melanoma	33	8.00 ± 3.14
Controls	51	5.98 ± 3.07

and concentration values of Thormählen positive melanogens of the melanotic group are clearly higher than in other groups. The group of hamsters with melanotic melanoma includes animals shortly after transplantation, when the tumour growth is macroscopically difficult to trace. This becomes evident when we plot the excretion of Thormählen positive melanogens versus days after transplantation (Fig. 5). This fact reduces the average values and has an unfavourable effect on other characteristics of the group.

Despite this, the values of excretion and/or concentration of Thormählen positive melano-

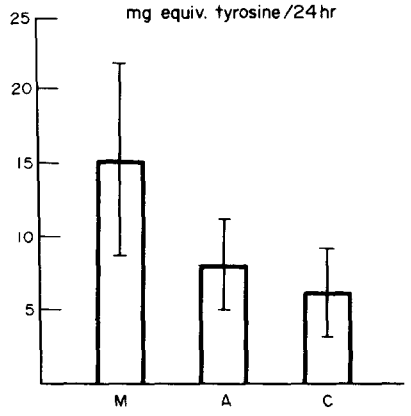

Fig. 3. Urine excretion of phenolic compounds, which react with diazotized p-*nitroaniline according to Gan* et al. (1975). *M—hamsters with melanotic melanoma; A—hamsters with amelanotic melanoma; C—hamsters without tumours (controls). The values are summarized numerically in Table 3.*

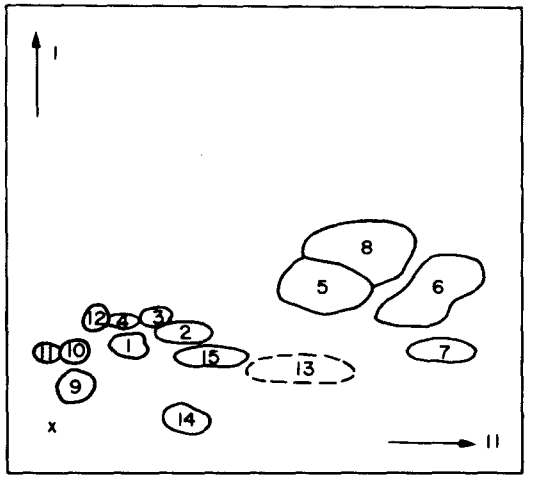

Fig. 4. Two-dimensional thin-layer chromatography of ethyl acetate extracts of hamster urine after the spraying with diazotized p-*nitroaniline in alkaline medium: 2—vanilmandelic acid; 3—m-hydroxyhippuric acid* (?); 4—p-*hydroxymandelic acid* (?); 5—*vanilpyruvic acid* (?); 6—*homovanillic acid; 13—mixture of* 5-*hydroxy*-6-*methoxyindolyl*-2-*carboxylic and* 6-*hydroxy*-5-*methoxyindolyl*-2-*carboxylic acids; 14—3,4-dihydroxyphenylacetic acid; 15—5-hydroxyindolyl*-3-*acetic acid. Compounds No.* 1, 7, 9, 10, 11, 12 *remained unknown. Compound No.* 13 *was present only in urine of hamsters with melanotic melanoma.*

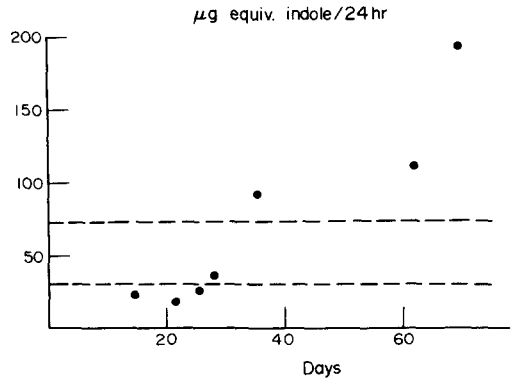

Fig. 5. Dependence of excretion of Thormählen positive melanogens on days after transplantation. Every point is an average of 4–5 measurement. Horizontal dotted lines represent mean ±S.D. of excretion of Thormählen positive melanogens in control hamsters.

gens, as well as of the compounds which give a positive diazo test, are significantly higher in hamsters with transplantable melanotic melanom ($P<0.01$) when compared with the values of excretion and/or concentration of Thormählen positive melanogens in hamsters with amelanotic melanoma and in control animals.

Thin-layer chromatography of acid ethyl acetate extracts of hamster urine showed the presence of at least 15 compounds and only one qualitative difference was noted. It was the presence of 5-hydroxy-6-methoxyindolyl-2-carboxylic and 6-hydroxy-5-methoxyindolyl-2-carboxylic acid in the urine of melanotic melanoma hamsters. These compounds were not found in the urine of hamsters with amelanotic melanoma or control hamsters.

The presence of vanillactic acid was not observed in any of the urines. This, in our opinion, was due to the low levels of vanillactic acid in these urines. We had previously detected the presence of this acid in hamsters with melanotic melanoma (unpublished results). The presence of 3,4-dihydroxyphenylacetic acid in the urine of every hamster is a very interesting finding: this compound is very sensitive to alkaline media and for that reason was not found in paper chromatograms subjected to long development in an alkaline system. In addition, this catechol-acid is destroyed during the purification steps of extraction into ethyl acetate, re-extraction by an alkali solution followed by acidification and renewed extraction into ethyl acetate.

Our results suggest that the presence of melanotic melanoma in hamsters causes increased formation and excretion of some phenolic acids. As this increase was not found in animals with amelanotic melanoma, the quantitative differences in excretion could be due to the pigment melanin, in tumours.

The only qualitative difference between the groups was the presence of the isomeric 5-hydroxy-6-methoxyindolyl-2-carboxylic and 6-hydroxy-5-methoxyindolyl-2-carboxylic acids in the urines of hamsters with melanotic melanoma. We cannot, however, exclude the possibility that the above mentioned acids are formed in hamsters with amelanotic melanoma and controls, although in such small amounts that we were unable to detect them by the present methods.

Qualitative and quantitative differences could be caused either by different metabolic processes in tumour tissue or by different interaction of tumours with host organism. Hamsters with transplantable melanoma seems to be a good model for further study of melanogenuria.

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Bacterial Infection and Acute Myeloblastic Leukaemia: An Analysis of Two Hundred Patients Undergoing Intensive Remission Induction Therapy

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Abstract—*A survey was undertaken to define the infectious problems which occurred during intensive treatment of acute myeloblastic leukaemia. Two-hundred patients, treated consecutively at a single hospital, were studied. Bacteraemia, pneumonia and wound infecions were the most common infectious problems; the most important bacterial pathogens were* Escherichia coli, Pseudomonas aeruginosa *and* Staphylococcus aureus. *The presence of severe neutropenia and/or falling granulocyte count were accompanied by a worse prognosis. Patients who had a temperature of at least 38°C at initial presentation had a significantly worse complete remission (CR) rate ($P<0.015$) and survival rate ($P<0.01$) than patients who were afebrile; this difference could not be explained by more frequent early death in the febrile group.*

Presence of fever at diagnosis, like age and platelet count, is thus an important prognostic factor. A scoring system, based on these diagnostic features, should help to identify those patients who, because of high probability of entering CR, should be aggressively treated. Conversely, the outlook for non-responding patients may be improved by the delivery of less intensive chemotherapy when probability of entering CR, based on criteria available within hours of admission, is very low.

INTRODUCTION

OVER the past decade a number of advances in the management of acute myelogenous leukaemia (AML) have been made as a result of more effective chemotherapy, better supportive care and the introduction of immunotherapy [1–3]. Despite small increases in survival, the disease remains almost universally fatal. Since the advent of fresh platelet transfusions has reduced the morbidity and mortality of haemorrhage [4], overwhelming infection during periods of neutropenia has become increasingly important as a cause of death [5]. It is well recognized that the risk of infection in patients with acute leukaemia is inversely proportional to the circulating granulocyte count at levels below 1000 cells/mm^3 and that delay in treating such infections may seriously hinder the likelihood of response [7]. Empirical treatment of febrile neutropenic patients with broad-spectrum antibiotics has therefore become more widely employed. Since the optimal design of empirical antibiotic regimes relies on a knowledge of common pathogens, documentation of the infectious problems during induction of first remission in AML is important. In this survey we have studied the pattern of bacterial infection seen during the initial intensive remission induction period, the importance of fever as a presenting feature, and the relative effectiveness of a variety of antibiotic combinations.

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MATERIAL AND METHODS

The study population consisted of 200 consecutive patients with AML, all treated at a single hospital from 1971 to 1975. The age range was 14–74 yr (mean age 49.6). Only four patients

had received significant previous treatment (usually with 6-mercaptopurine or methotrexate). Unequivocal diagnosis of AML or a variant (including acute promyelocytic, acute myelomonoblastic, acute erythroblastic or acute monoblastic leukaemia) was confirmed in all cases by bone marrow puncture.

On admission to hospital, routine surveillance cultures were performed on all patients. These included swabbing of nose and throat; culture of urine and blood; and culture of stool specimens if diarrhoea was present. Infected lesions were always swabbed. Routine cultures for anaerobic organisms were not performed unless indicated clinically. As well as haematological and biochemical tests, a chest X-ray was always obtained. Almost all patients received intensive chemotherapy, either with one or more courses of cytosine arabinoside and daunorubicin, or with cytosine arabinoside, adriamycin, vincristine and prednisolone, in an attempt to achieve a complete remission (CR). Some patients later received other drugs in addition; these included vincristine, prednisolone, 6-thioguanine and cyclophosphamide. Most patients became severely neutropenic (<200 cells/mm^3) during the first few weeks after treatment was commenced. If the patient became febrile, all cultures were repeated and further chest X-rays were taken throughout the febrile period; at least one further set of blood cultures (2–3 bottles per set) was obtained before instituting antibiotic treatment.

The chief criterion for administration of antibiotics was the presence of fever of at least 38°C on two consecutive occasions, separated by a 2–4 hr interval. In patients who had fever which was thought to be due to administration of blood products or a drug allergy, antibiotic therapy was withheld until it seemed likely that the continued fever could no longer be ascribed to these factors. If bacteriological documentation of infection was obtained, these data dictated further therapy. If the organisms were sensitive to agents used in the empirical regime, these drugs were continued. In the absence of bacteriological proof, the antibiotics were continued if fever defervescence was judged to be satisfactory. Administration of antibiotics continued for 5 days after fever had returned to normal. If there had been no response to the fever at 5 days, the empirical antibiotic regime was discontinued and an alternative regime was commenced. Response to antibiotic therapy was considered successful if fever abated within five days of institution of antibiotic therapy and symptoms and signs of infection had cleared by this time. However, in some clinical situations, healing of infected lesions, such as large abscesses or radiologically evident pneumonias, though clearly occurring by five days, was not complete. If such lesions continued to improve and eventually showed complete resolution, these were also judged to be successful responses to the empirical antibiotic therapy. If fever defervescence was obtained but bacterial cultures were still positive, this was judged to be a failure. Other failures of therapy were judged to have occurred when there was no fever defervescence, when febrile patients died after an adequate trial (48 hr) of antibiotic therapy, or if cultures were positive even in the absence of the fever at any point during therapy. Equivocal responses were considered as treatment failures. Episodes of presumed infection were considered evaluable unless patients died within 24 hr of commencing antibiotic therapy.

Laminar airflow rooms were not employed and patients were almost always nursed on an open ward. In a small number of cases (34 courses of antibiotic therapy out of a total of 619), evaluation was not possible because of inadequate data.* All statistical analyses were performed using the chi-squared test with Yates' correction (1 degree of freedom), unless explicitly stated otherwise.

RESULTS

Frequency of infection during remission induction of AML

Only 5 of 200 patients remained afebrile throughout the entire period of remission induction. The average number of antibiotic treatment courses during this period was 3.1 patient. Positive bacteriological culture was obtained on 398 occasions (Table 1). The commonest bacterial species (all sites) were *E. coli*, *Staph. aureus* and *Ps. aeruginosa*. Although the frequency of infection with most organisms remained constant throughout the study period, there was an unusually high incidence of pseudomonas infections in 1972–1973.

Bacteraemia was encountered on 60 occasions; organisms causing bacteraemia were similar to those causing other infections (Table 2). *Staph. albus* bacteraemia was only considered significant in the 17 cases in which the organism was grown from all bottles of culture medium on two consecutive occasions within 48 hr. *Pseudomonas* and *Klebsiella* were equally frequent causes of Gram-negative bacteraemia as *E. coli* and *Staph. albus* was more commonly cultured from blood than expected from its incidence at

*Failure to evaluate these episodes was chiefly due to inadequate records of cause of fever (17 episodes) or outcome (12 episodes).

Table 1. Positive bacterial cultures (all sites included) in 200 patients with AML at St Barholomew's Hospital over a 5-yr period

	E. Coli	*Pseudomonas*	*S. Aureus*	*β Haemolytic strep.*	*Enterococcus*	*S. Albus*
1971	12	2	18	8	4	5
1972	21	10	19	11	6	5
1973	27	27	13	10	11	10
1974	22	3	15	6	9	7
1975	26	5	15	7	5	9
Total	108	47	80	42	35	36

Others (totals)

Klebsiella spp.	9	*Cl. Welchii*	2	*Niesseria meningitidis*	1
Proteus spp.	9	*Pneumococcus*	4	*Salmonella typhimurium*	1
Haemophilus spp.	6	*Str. viridans*	3	*Enterobacter liquifaciens*	2
Micrococcus	9	*Mycoplasma pneumoniae*	1	*Bacteroides* spp.	1

other sites. In 14 of the 17 *Staph albus* bacteraemias, an intravenous catheter was present. Three episodes of multiple-organism bacteraemia were seen, all of which were fatal. Fatal *Candida* septicaemia was seen on only one occasion.

Miscellaneous infections included pulmonary tuberculosis (two cases), Salmonella osteomyelitis (one case) and two episodes of acute appendicitis with fulminant septicaemia, both of which were fatal. Single cases of fatal brain abscess (*E. coli*), fatal lung abscess (*K. aerogenes*) and non-fatal infected marrow puncture site (*Staph. aureus*) were also seen.

Table 2. Organisms causing bacteraemia in 200 patients with AML over a 5 yr period

Organism	No. of infections
Staph. albus	17*
E. coli	9
Klebsiella spp.	9
Pseudomonas aeruginosa	8
Staph. aureus	6
β haemolytic *Streptococci* and *enterococci*	3
Pneumococcus	2
Strep. viridans	2
Enterobacter spp.	2
Candida spp.	1
Bacteroides	1

**Staph. albus* bacteraemia was only considered significant in the 17 cases in which the organism was grown from all bottles of culture medium on two consecutive occasions within 48 hr.

Fever, remission and survival

The presence of fever on admission to hospital was an adverse prognostic sign (Table 3). A CR was obtained in 39/93 (42%) of patients who were afebrile (<37°C) on admission, whereas a CR was obtained only in 26/107 (24%) of patients who were febrile (>37°C) on admission ($0.012 < P < 0.015$). This was *not* chiefly due to more frequent early deaths in the febrile group, since the CR rate in patients surviving at least four weeks from diagnosis was 39/74 (53%) of initially afebrile patients and 26/74 (35%) of initially febrile patients ($P < 0.05$). Thus patients who were febrile on admission retained their poor prognosis *even if they survived their initial infection*. A slightly greater number of initially febrile patients succumbed, however, during the first four weeks (33/107 initially febrile patients compared to 17/93 in the afebrile group: $P > 0.05$).

Survival was also significantly different in the two groups. Initially afebrile patients had a mean survival of 8.6 months whilst initially febrile patients had a mean survival of only 5.0 months (all patients included, whether in remission or not: $P < 0.01$).

Patients who were febrile on admission to hospital also differed haematologically from afebrile patients, but not in the important prognostic variables of age and initial platelet count (Table 4). Mean initial white blood count (WBC) and absolute blast count (BC) were significantly lower for afebrile patients (WBC: $23.2 \pm 4.7 \times 10^3/mm^3$ vs $55.7 \pm 10.2 \times 10^3/mm^3$; BC: $15.9 \pm 4.2 \times 10^3/mm^3$ vs $37.7 \pm 6.9 + 10^3/mm^3$). However, despite this correlation, the influence of initial WBC and BC on probability of CR was not statistically significant (WBC: $P > 0.12$; BC: $P > 0.20$, log rank test). Initially febrile patients who achieved a CR had lower presenting WBC's than febrile patients who failed to achieve CR ($39.4 \pm 9.6 \times 10^3/mm^3$ vs $59.5 \pm 13.0 \times 10^3/mm^3$) but this trend was not statistically significant. The same trend was

Table 3. Prognostic importance of fever on first admission to St. Bartholomew's hospital with newly-diagnosed AML

	Febrile patients (>37°C)	Afebrile patients (<37°C)	Significance (*P*)
Total patients:	107	93	
Probability of achieving CR:	26/107 (24%)	39/93 (42%)	<0.015
Deaths within first 4 weeks:	33/107 (31%)	17/93 (18%)	>0.05
Probability of CR excluding deaths within 4 weeks:	26/74 (35%)	39/74 (53%)	<0.05
Survival (months) whether in remission or not:	5.0±0.8*	8.6±0.9*	<0.01

*Mean ± 1 standard error.

Table 4. Significant characteristics of afebrile and febrile groups

	Febrile patients	Afebrile patients
Age in years	49±1.9*	48.5±1.8
Initial platelet count ($\times 10^6$/mm^3)	74.3±10.1	73.4±9.9
Initial white blood cell count ($\times 10^3$/mm^3)	55.7±10.2	23.2±4.7 ($P<0.007$)
Initial absolute blast cell count ($\times 10^3$/mm^3)	37.7±6.9	15.9±4.2 ($P<0.008$)

*Mean ± 1 standard error.

observed for presentation BC ($29.6 \pm 9.2 \times 10^3$/mm^3 vs $37.4 \pm 8.6 \times 10^3$/mm^3).

Influence of age and initial platelet count

As noted elsewhere for substantially the same group of patients [8], both age and initial platelet counts were important predictors of response to antileukaemic therapy. Complete responses were obtained in 7/10 (70%) of patients under 20 yr, 29/61 (47.5%) of patients aged 20–40 yr, 23/76 (30%) of patients aged 41–60 yr and 12/53 (23%) of patients over 60 yr. Mean age of all complete responders was 39.8 yr, whilst that of non-responders was 46.8 yr, ($P < 0.01$, log rank test). *CR* was achieved in 42% of patients presenting with platelet counts above 20,000/mm^3 and only 27% of patients whose presenting platelet counts were below this figure ($P < 0.05$, log rank test). Age, probability of fever on admission and platelet count were independent; mean age of all initially afebrile patients was 48 yr, whilst that of all initially febrile patients was 45.5 yr ($P > 0.5$). Mean platelet counts were almost identical in the febrile and afebrile groups (Table 4).

Influence of granulocyte counts during therapy

Likelihood of successful response to antibiotic therapy was influenced both by the initial granulocyte count (at the onset of fever) and the change in granulocyte count during the febrile episode (Tables 5 and 6). Two hundred and fifty-four episodes of fever (in which absolute granulocyte counts were available on the first and last day of the febrile episode) were analysed. Overall response rate in this group was 61%, which was not significantly different from the response rate observed for the complete group. Of 20 episodes which proved fatal, 15 occurred in patients whose granulocyte count at the start of the fever was less than 100/mm^3. With initial granulocytopenia to less than 100 cells/mm^3, success rate was only 45%, whereas success rates of over 70% were seen if the initial granulocyte count was greater than 100 cells/mm^3. Favourable response was most likely if a rise in granulocyte count occurred during the course of the treatment. In 68/78 (88%) of such courses, there was a good response, regardless of the initial granulocyte count (Table 6). If granulocytes fell during therapy, only 64/109 (59%) of courses were successful. In 45 cases, febrile episodes occurred during periods in which granulocytes were totally undetectable throughout the course of treatment; 11 (24%) of these episodes proved fatal. Most infections occurred during extreme periods of marrow aplasia; the median granulocyte count at which fever was first noted to be present was 140 cells/mm^3 (standard error ± 24 cells/mm^3).

Table 5. Influence of initial granulocyte count on response to antibiotic therapy

Absolute granulocyte count (cells/mm^3)	Type of response: Success		Non-fatal failure		Fatal failure	
	No.	%	No.	%	No.	%
<100	54	45	50	42	15	13
100–499	49	74	14	21	3	5
500–999	18	82	4	18	—	—
>1000	34	72	22	23	2	4

Number of courses analysed: 254.
Fatalities: 20.

Table 6. Importance of change in granulocyte count during antibiotic therapy

	Type of response: Success		Non-fatal failure		Fatal failure	
	No.	%	No.	%	No.	%
Granulocytes ↑ during fever	68	88	8	10	1	1
Granulocytes ↓ during fever	64	59	37	34	8	7
Granulocytes entirely absent during courses	8	18	26	58	11	24

*Table 7. Bacterial infection in patients with AML: response by site in documented cases**

	Success No.	(%)	Fatal failure No.	(%)	Non-fatal failure No.	(%)
Septicaemia — Gram positive	22	(76)	7	(24)	0	(0)
Septicaemia — Gram negative	12	(46)	14	(54)	0	(0)
Pneumonia — X-ray only	36	(62)	19	(33)	3	(5)
Pneumonia — X-ray + positive sputum culture	33	(70)	12	(26)	2	(4)
Wound infection	43	(88)	3	(6)	3	(6)
Urinary infection	34	(72)	7	(15)	6	(13)

*Additional positive cultures of doubtful significance were obtained from throat and nose swabs, as follows: Haemolytic *Streptococci* (20 patients), *E. coli* (15 patients), *S. aureus* (31 patients), *Pseudomonas aeriginosa* (5 patients), *Enterococcus* (3 patients). *Mycoplasma pneumoniae* (1 patient), *Proteus* spp. (1 patient).

Site and prognosis of infection in AML

A total of 69 patients died during the study period; in 62 of these, infection was considered to be a significant contributing factor. Regardless of the empirical regime used, the probability of successful response was dependent on the site of infection (Table 7). The worst prognosis was seen in patients with Gram-negative septicaemias (fatal in 54% of 26 cases) and pneumonias (32% fatal in 105 cases). The presence of positive sputum-culture during the episode of pneumonia did not influence prognosis. Patients with documented Gram-positive bacteraemia had a significantly higher survival rate ($P<0.05$) than those with a Gram-negative bacteraemia. Failure of response to antibiotic therapy was usually fatal in pneumonia and invariably fatal in septicaemia. Failure of soft tissue or surface lesions to respond to antibiotic therapy was unusual (6/49 failures). All six soft-tissue failures were associated with perianal or anorectal abscess, fistula or fissure. Twelve such lesions were documented; six were successfully treated with antibiotics with or without surgical drainage.

Empirical antibiotic regimes

The choice of empirical antibiotic combinations reflected the availability of newer and more promising antibiotics over the past 5 yr (Table 8). All antibiotics were routinely given by the intravenous route, with the exception of the first two pairs of agents, which were usually

Table 8. Five yr empirical antibiotic therapy in febrile neutropenic patients with AML

Empirical combination	Year of most frequent use	Number treated	Success rate (%)
Ampicillin cloxacillin }	1971	97	47
Erythromycin/chloramphenicol }		94	53
Polymyxin B/flucloxacillin	1972	72	57
Gentamicin/cephalothin	1973	91	62
Gentamicin/carbenicillin	1974	106	65
Tobramycin/carbenicillin	1975	47	68
		507	58

given by mouth. The overall response rate (all combinations, all evaluable treatment courses included) was 58%, with a gradual improvement in response rate during each year of the study period. Since fresh platelet transfusions and granulocyte transfusions have been available in this unit throughout the whole period, the improvement in response rate is unlikely to be due to better supportive care in recent years.

DISCUSSION

As with previous studies [5, 9, 10], infection during periods of neutropenia was the chief cause of death in our patients undergoing remission induction for AML. Although patients who were febrile on admission were at an increased risk of death during the first four weeks after diagnosis, this trend was not statistically significant, unlike the study of Yates and Holland [11]. In our patients, presence or absence of fever on admission was clearly of prognostic importance, even when all deaths within the first four weeks (usually the result of infection) were excluded from analysis. In the study by Carey *et al.*, patients who were febrile at presentation were less likely to enter CR, but the reasons were unclear [12]; in particular, it was not stated whether such patients failed to enter CR because of more frequent early death. In the present study, the better prognosis of the initially afebrile patients, both for remission ($P<0.015$) and survival ($P<0.01$), remain largely unexplained, and cannot be accounted for on the basis of lower mean age or higher initial platelet count.

Since initial blast count in the peripheral blood was lower in this group ($P<0.008$), such patients presumably had a smaller tumour burden; however, initial total blast count and total white blood count are not usually considered to be major risk factors [13] and their influence in the present study did not reach statistical significance. An alternative explanation is an increased likelihood of reinfection in patients who were initially febrile since such patients may harbour pathogenic organisms more frequently.

Particularly lethal were the Gram-negative septicaemias and pneumonias, although the death rate from pneumonia in this series was approximately half the mortality rate noted by Sickles *et al.* in a group of 68 patients with acute leukaemia [14], presumably reflecting the greater efficacy of prompt antibiotic treatment when given almost immediately a fever of 38°C was observed in any neutropenic patient. In the study by Viola [9], pneumonia constituted four of 33 leukaemia deaths, but pneumonia with bacteraemia was responsible for a further ten deaths. In a study of 22 cases of perianal or anorectal complications of leukaemia, Sehdev *et al.* reported a fatality rate of 7 deaths out of 18 episodes in patients with acute leukaemia [15]. Our experience was similar, with three fatalities amongst 12 patients.

Since bacteriological documentation of infection may require two or more days, the use of empirical antibiotic therapy for febrile episodes during periods of neutropenia is now widely accepted [16]. Many empirical combinations have been reported [17—19] and treatment has usually been instituted after 48 hr if fever persists. Our practice since 1971 has been to wait no longer than four hours, providing that obvious non-infectious causes of fever can be excluded. The potentially dangerous drawback of low bacteriological confirmation rate has probably been offset by the gradually improving response rates, especially since the more widespread use of high doses of carbenicillin. Other groups have also confirmed relatively good response rates in neutropenic patients treated with combinations which include this antibiotic [17, 20].

In this hospital the chief agents identified during the 5 yr study period were *E. coli* (108), *Staph. aureus* (80) and *Ps. aerguinosa* (47). *Pseudomonas* infections appeared to be particularly common in 1972–1973, and this changing trend of bacterial flora has been noted elsewhere [21]. It seems logical therefore to include an aminoglycoside antibiotic in any empirical regime. Because of the continuing and serious danger of pseudomonas infections, a combination of an aminoglycoside such as gentamicin or tobramycin with large doses of carbenicillin, should provide adequate antibacterial cover. Good evidence now exists for therapeutic synergism between gentamicin and carbenicillin [22]. Since tobramycin is superior to gentamicin for many strains of *pseudomonas in vivo* [23] (including many gentamicin-resistant strains) [24], it should perhaps be preferred. At present, there are no conclusive data in man which have confirmed that this theoretical advantage is clinically important in neutropenic patients. Concurrent administration of aminoglycosides and high doses of cephalosporins has been implicated as a potential cause of nephrotoxicity [25, 26] and the combination should be used with caution. A large randomised cooperative study has shown that the combination of cephalothin and gentamicin was significantly more nephrotoxic than other equally effective antibiotic regimes [27].

Although empirical antibiotic therapy in febrile neutropenic patients with AML and other malignant diseases has now been widely accepted as proper management, the ideal of a broad-spectrum, effective, non-toxic and cheap antibiotic regime has not yet been realised. Prophylaxis with gut sterilising antibiotic combinations may decrease the likelihood of infection [28], but fails to abolish it entirely. Likewise, the routine use of laminar airflow rooms remains controversial with regard to both cost and value [29]. For established infection, possible alternative measures include therapeutic granulocyte transfusions in patients who are failing to respond to antibiotic therapy [30, 31], the use of transfer factor [32], and the administration of more effective doses of antibiotics already known to be of value by computing dosage on the basis of age, weight, sex and renal function [33]. It is known that satisfactory response to gentamicin therapy in non-neutropenic patients depends on the achievement of appropriate circulating levels [34]; conventional doses may be insufficient to achieve such levels. Closer attention to early treatment of febrile episodes with adequate doses of appropriate antibiotics (with monitoring where necessary) should lead to a greater recovery rate in infected neutropenic patients with AML and therefore an increased likelihood of achieving a complete remission. Moreover, initial assessment of simple presentation characteristics such as age, platelet count and presence of fever may permit the development of a risk-factor scoring system in patients with AML. Since early death is often due to overtreatment of patients in whom achievement of a complete remission is highly unlikely, such a scoring system may allow such patients to be treated less aggressively without aiming for complete remission but with greater emphasis on supportive care. This approach should improve the median survival of non-remitting patients who still comprise approximately one-half of all patients with AML, whilst in no way compromising aggressive attempts to induce a more durable and complete remission in patients who are young, afebrile on admission and not grossly thrombocytopenic.

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The Immunostimulatory Activity of 3-(*p*-Chlorophenyl)-2,3-Dihydro-3-Hydroxythiazole [3, 2-α]-Benzimidazole-2-Acetic Acid (NSC 208828)*

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Abstract—*A preliminary characterization of* 3-(p-*chlorophenyl*)-2,3-*dihydro*-3-*hydroxythiazolo* [3,2-α]-*benzimidazole*-2-*acetic acid was conducted in mice employing tumorous and non-tumorous systems. Single doses of this chemical markedly increased the primary and secondary anti-SRBC response after optimal immunization and raised the GvH-inducing capacity of splenocytes whereas no effects were seen on the response to a T-independent antigen.*

Immunostimulatory doses of the drug did not modify colloidal carbon clearance, macrophage non-specific cytotoxicity towards lymphoma cells or K cell activity in the spleen. Single injections with this agent increased survival in L 1210*Ha leukemia-bearing mice when administered in conjunction with irradiated tumor cells and increased anti-leukemia resistance to subsequent tumor challenges in animals primed with inactivated leukemia cells. Single drug doses* 1 day *after tumor transplantation exerted also a potent antimetastatic effect in the Lewis lung carcinoma system.*

INTRODUCTION

MUCH interest is currently focused on non-specific immunomodulators in view of the favourable results obtained through their use in the treatment of experimental tumors and of at least a number of human malignancies. Attention towards this type of agents has additionally been spurred by preliminary indications of their potential in other conditions in which a disordered or insufficient immune reactivity is believed to be of pathogenic importance such as rheumatic diseases, recurrent or chronic infections, primary immunodeficiencies, etc. [1, 2]. The bacterial origin of the immunostimulants more widely employed in the clinic, i.e. BCG and *Corynebacterium parvum* (*C. parvum*), in addition to being responsible for a number of side effects [3–5], has also resulted in problems of standardization and in difficulties in the analysis of their mode of action. There is thus the need for a larger number of active compounds having defined chemical characteristics, greater ease of employ and different mechanisms of action at the immunocyte subpopulation levels.

This report describes the results obtained in the preliminary characterization of 3-(*p*-chlorophenyl)-2,3-dihydro-3-hydroxythiazole (3,2-α)-benzimidazole-2-acetic acid (NSC 208828, Fig. 1), a synthetic compound possessing definite immunomodulatory capacity.

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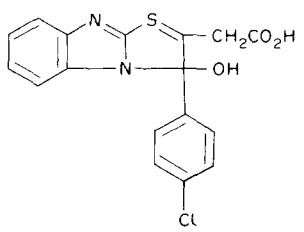
MATERIAL AND METHODS

Animals

Mice of both sexes of the following strains were used: C57Bl/6; C3H, B6D2F$_1$ (C57Bl/6 × DBA/2) and CD2F$_1$ (BALB/c × DBA/2). The animals were obtained from Charles River, Italy (Calco, Italy) and weighed 20–22 g at the start of the experiments.



NSC 208828

Fig. 1. Chemical structure of NSC 208828.

Drugs

NSC 208828, kindly provided by Dr. E. Alburn (Wyeth Laboratories, Philadelphia, Pa.) was freshly suspended in sterile 0.9% NaCl solution containing 1% Tween 80 and administered intraperitoneally (i.p.). Cyclophosphamide (Cy), supplied by Dr. H. J. Wood (DRD, NCI, Bethesda, Md) was freshly dissolved in saline. Suspensions of formalin-killed *C. parvum* (lot 086 P, Wellcome Research Laboratories, Beckenham, England) were diluted with saline and injected intravenously (i.v.) in the dose of 0.7 mg/mouse.

Tumors

The spontaneously metastasizing Lewis lung (3LL) carcinoma was maintained and transplanted intramuscularly (i.m.) in C57Bl/6 mice, its strain of origin, the standard inoculum being 2.10^5 viable cells; the extent of pulmonary metastatization was assessed as previously described [6]. The L1210 Ha Leukemia, i.e. a subline previously described as being immunogenic in compatible hosts [7], was maintained by weekly i.p. passages in syngeneic DBA/2 mice (H-2^d) whereas for experimental tests it was transplanted in compatible $CD2F_1$ (H-2^d/H-2^d) or allogeneic C3H (H-2^k) mice.

Response to sheep erythrocytes and type III pneumococcal polysaccharide

Conditions for the assay of primary spleen hemolytic plaque-forming cells (PFC) after the i.p. injection of 10^8 sheep erythrocytes (SRBC) were as previously described [6]; for secondary responsiveness mice were given two equal SRBC inocula 10 days apart and indirect PFC developed using optimal dilutions of commercially available rabbit antiserum to mouse gamma globulins. For type III pneumococcal polysaccharide (S III, a gift of Dr. P. J. Baker, NIAID, NIH, Bethesda, MD), the optimally immunizing dose of 0.5 μg was administered i.p. and specific anti-SIII PFC measured as detailed elsewhere [7].

Graft vs Host (GvH) assay

GvH was assessed by the popliteal lymphnode weight gain assay [8]. Recipient $B6D2F_1$ (H-2^b/H-2^d) female mice immunodepressed 24 hr before with 100 mg/kg i.p. Cy, were injected into the right hind footpad with a suboptimal number (3×10^6/0.05 ml) of splenocytes from control or drug-treated C3H mice of the same sex. Equal numbers of formol-inactivated cells were injected contralaterally. Both popliteal lymph nodes were collected 7 days later and the ratio between the weight of the right and left nodes (lymphnodal index) taken as a measure of the severity of GvH [9].

Clearance of colloidal carbon

The procedure of Biozzi *et al.* [10] was employed injecting $CD2F_1$ mice with 320 mg/kg i.v. of colbidal carbon (Pelikan ink, lot C11/1431/A).

Macrophage cytotoxicity

The cytotoxic capacity of purified peritoneal macrophages from $CD2F_1$ mice towards exponentially growing SL2 and TLX9 lymphoma cells was evaluated after 48 hr incubation by cell counting of the surviving lymphoma cells and by their uptake of tritiated thymidine following experimental conditions previously reported [11].

K cell activity assay

Technical conditions employed to evaluate in $CD2F_1$ mice spleen K cell activity against chicken erythrocytes as targets were as detailed previously [12].

Statistical analysis

Statistical significance of the differences in mean survival times (MST) was assessed by the Mann–Whitney U test, each experimental group consisting of at least 10 mice. Statistical significance for the other parameters was established by Duncan's new multiple range test with experimental groups consisting of at least 6 animals.

RESULTS

Effects of NSC 208828 on immune reactivity

The capacity of single i.p. injections of NSC 208828 (100 mg/kg) to influence the primary response of $CD2F_1$ mice to SRBC was firstly investigated, administering the drug on various days in relation to antigen and counting the PFC 4 days after SRBC, i.e. at the peak response in these conditions. Significant increases in PFC numbers were

Table 1. *Time-dependency of the effect of NSC* 208828 *on the primary anti-SRBC response*

Day of treatment	PFC/Spleen
—	24,230 (17,420–33,710)
−3	26,340 (19,370–35,560)
0	27,180 (12,840–54,730)
+1	62,070* (56,160–68,600)
+2	68,050† (55,710–83,460)

$CD2F_1$ mice (6 per experimental group) were injected with 10^8 SRBC i.p. on day 0; drug (100 mg/kg i.p.) was given at various times relative to immunization and assay was performed on day 4. Results presented are geometric means (in parentheses ±S.E.) after logarithmic transformation of the data. Values shown are means of 3 experiments.

*$P<0.05$; †$P<0.01$.

Table 2. *Effect of different doses of NSC* 208828 *on the primary anti-SRBC and anti-S* III *responses*

Dose (mg/kg i.p.)	PFC/Spleen	
	anti-SRBC	anti-S III
—	24,500 (20,010–30,000)	8,700 (6,900–10,300)
25	30,700 (24,100–37,450)	8,230 (6,710–9,930)
50	62,900* (44,740–81,530)	8,460 (5,130–11,420)
100	77,270† (56,900–93,870)	9,275 (9,390–10,250)
150	63,050* (56,900–79,990)	9,550 (8,630–10,340)
200	19,960 (16,360–24,350)	N.T.

$CD2F_1$ mice (6 per experimental group) were injected i.p. with 10^8 SRBC or 0.5 μg S III on day 0, drug was given on day 2 and the assay performed on day 4 and 5 for SRBC and S III, respectively. Values shown are means of 3 experiments.

*$P<0.05$; †$P<0.01$.

observed (Table 1) only when the drug was given after immunization, pre or concomitant treatments being inactive in these conditions. Table 2 shows the dose–response relationship obtained with drug treatments performed 48 hr after SRBC; whereas doses of 25 or 200 mg/kg did not produce significant changes in PFC values, the injection of 50, 100 or 150 mg/kg resulted in a trebling of the PFC response. A similar increase in PFC values was present also when the assay was performed 7 days after SRBC and approximately a doubling in the total number of spleen direct and indirect PFC was seen when 100 mg/kg of the chemical was injected 48 hr after a second SRBC challenge and the test was performed after a further 2 days (data not shown). The effects of this compound on the primary response to a T-independent antigen such as S III was next investigated. As presented in Table 2, treatments with this agent capable of markedly augmenting the anti-SRBC response were not significantly effective in modifying the number of peak (day 5) anti-S III PFC in the spleen, and a follow-up of the response at later days also failed to evidentiate significant differences between control and drug-treated animals.

In order to obtain informations on the activity of NSC 208828 on cellular reactivities, its effect on the GvH-mounting capacity of lymphoid cells was studied. As shown in Table 3, whereas a single injection of 50 mg/kg one day before sacrifice of the cell donor resulted in a modest and not significant increase in the splenocyte capacity to induce a GvH, a clear activity was seen with 100 or 150 mg/kg, the percent increases in lymph-

Table 3. *GvH reactivity of NSC* 208828-*treated spleen cells*

Drug (mg/kg i.p.)	Popliteal lymph node index	Increase (%)
—	1.6±0.1	—
50	2.2±0.1	37
100	2.6±0.2*	63
150	2.9±0.3†	81

3×10^6 splenocytes of C3H female mice treated 1 day before with NSC 208828 were injected in the footpad of $B6D2F_1$ female mice which had been immunosuppressed with 100 mg/kg i.p. of cyclophosphamide 24 hr previously.

Recipient mice (6 per group) were sacrificed 8 days later.

*$P<0.05$ vs control.

†$P<0.01$ vs control.

Table 4. *Effect of NSC* 208828 *on allogeneic tumor growth*

Dose (mg/kg i.p.)	MST*	D/T†
—	10	2/10
25	7.7±0.5	4/10
50	12.3±1.0	7/10‡
100	13.2±0.9	8/10§
150	11.6±1.9	6/10

C3H female mice were injected with NSC 208828 one day before inoculation with 15×10^6L 1210 Ha leukemia cells.
*Mean survival time in days±S.E.
†Dead with leukemia over total number of tumor transplanted mice.
‡$P<0.05$ vs control.
§$P<0.01$ vs control.

nodal index over controls being in fact 63 and 81%, respectively. Studies were also conducted to investigate the effects of this chemical on allogeneic tumor growth. As illustrated in Table 4 by representative data, the treatment with this drug of C3H recipients 24 hr before the transplant of incompatible L1210 Ha leukemia cells was followed by an increase in the number of progressive tumor takes evident at all drug doses tested, although the effect reached statistical significance only after doses of 50 or 100 mg/kg. In view of the effects of various immunomodulators on macrophages, the functional capacity of these elements in animals treated with NSC 208828 was explored. No significant modifications in colloidal carbon clearance were however detected after the administration of otherwise active doses of the compound; for instance, values of 0.026 and 0.029 were calculated for the granulopectic index in control and $CD2F_1$ mice given 100 mg/kg i.p. of this agent 3 days before assay. In the same conditions a value of 0.043 ($P<0.05$) was measured in mice given 0.7 mg i.v. *C. parvum*, employed as positive control agent. Furthermore, purified peritoneal macrophages collected from $CD2F_1$ mice 7 or 15 days after the administration of 100 mg/kg NSC 208828 did not exhibit any significant cytotoxic activity towards cultured SL2 or TLX9 lymphoma cells as assessed either by tumor cell counts or by measuring the incorporation of radiolabelled thymidine in lymphoma cells. In the same testing conditions, the i.v. injection of 0.7 mg *C. parvum* resulted in 80–90% growth inhibition of the target tumor cells. Lastly, the effect of this drug on spleen K cell activity was investigated employing again

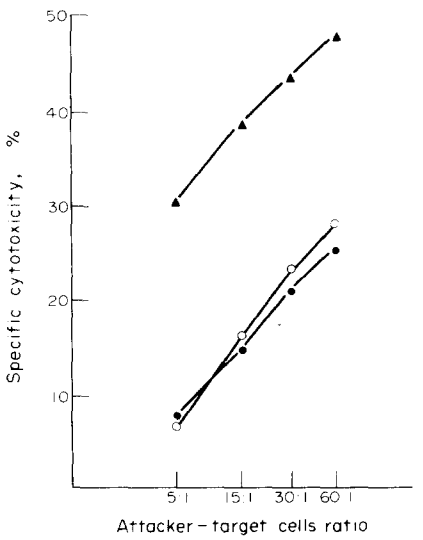


Fig. 2. *Spleen K cell activity in* $CD2F_1$ *mice* 7 days *after treatment with NSC* 208828 (100 mg/kg *i.p.*, ○———○), C. parvum (0.7 mg/*mouse i.v.*, ▲----▲) *or saline* (●———●).

C. parvum as a positive reference compound [1]. As shown in Fig. 2 where the data of one representative experiment performed testing the spleen cells 7 days after the injection of 100 mg/kg NSC 208828 are presented, no significant stimulation of K cell activity in respect to controls could be found. Similarly negative results were observed with drug doses ranging from 25 to 200 mg/kg and testing the splenocytes from 1 to 15 days after treatment.

Antitumoral effect of NSC 208828

The capacity of this agent in conjunction with a tumor cell vaccine to increase the survival of $CD2F_1$ mice subsequently challenged with graded numbers of the compatible L1210 Ha leukemia was firstly examined. Table 5 shows that single injections of 100 mg/kg of this drug could potentiate the protective effect exerted by the inactivated leukemia cells as evidenced by the significant extra increase in survival found when a challenge of 10^6 live leukemia cells was used and by the obtainment of a consistent proportion (30–50% in 4 such experiments) of cures (i.e. over 90 days survivors) when the challenge was of 10^5 L1210 cells. No significant increase in MST in respect to untreated controls was seen when 100 or 150 mg/kg of the drug was given on day 1 or on days 1–4 to $CD2F_1$ hosts transplanted with 10^4 or 10^3 L1210 cells i.p. or s.c. In contrast an antitumoral activity was seen when NSC 208828 was given together with 10^7 X-rayed leukemia cells i.p. 24 hr after the s.c. transplant of

Table 5. Survival of CD2F$_1$ mice given different inocula of L1210Ha leukemia after immunization with irradiated leukemia cells and treatment with NSC 208828

10^5 X-rayed L1210Ha cells i.p. Day 0	NSC 208828 (100 mg/kg i.p.) Day 1	No. L1210Ha cells i.p. Day 7	MST	D/T
−	−	10^7	6.3±0.1	10/10
−	+	10^7	6.5±0.3	10/10
+	−	10^7	7.2±0.2	10/10
+	+	10^7	8.9±0.3*	10/10
−	−	10^6	7.5±0.2	10/10
−	+	10^6	7.0±0.1	10/10
+	−	10^6	9.9±0.5*	10/10
+	+	10^6	13.4±0.7†	10/10
−	−	10^5	8.9±0.4	10/10
−	+	10^5	9.4±0.6	10/10
+	−	10^5	14.2±0.8*	10/10
+	+	10^5	17.0±0.8†	7/10

*$P<0.05$ vs untreated controls.
†$P<0.05$ vs X-rayed cells alone.

Table 6. Survival in L1210Ha bearing CD2F$_1$ mice treated with 2×10^7 irradiated leukemia cells and NSC D208828

Day 0 10^4 L1210Ha cells s.c.	Day 1 2.10^7 x-rayed L1210Ha cells i.p.	Day 1 NSC D208828	MST
+	−	−	12.0±0.5
+	+	−	15.0±0.7*
+	−	+	12.3±0.6
+	+	+	19.7±1.0†

NSC D208828 was given in the dose of 100 mg/kg i.p.
*$P<0.05$ vs control.
†$P<0.05$ vs X-rayed group.

10^4 cells of the immunogenic L1210 Ha subline (Table 6).

A clearer antineoplastic activity was on the other hand seen in the 3LL carcinoma system, in which single injections of the compound given 1 day after tumor transplant produced significant reductions in both the number and weight of the lung metastases although no detectable effects were exerted on the weight of the primary i.m. tumor (Table 7). In these conditions, the administration of 25 mg/kg of the compound showed a significant antimetastatic effect in only 1 of 2 experiments, whereas consistent results were observed with 50 or 100 mg/kg and the highest activity was found with a dose of 150 mg/kg, a treatment which also resulted in the absence of detectable lung metastases in 40–60% of the animals in 3 experiments. It may be noted that using the same treatment conditions, an optimal dose of 3 mg/kg i.p. of Levamisole, i.e. a dose previously shown to possess immunostimulant activity [6], was ineffective.

DISCUSSION

An immunostimulatory capacity for NSC 208828 has previously been advanced by Fenichel *et al.* [13] on the basis of its anti-metastatic activity in the 3LL system, which was absent in previously immunodepressed mice, and of its capacity to increase circulating T lymphocytes in rats. Our findings that single doses of this chemical can markedly stimulate in mice the primary and secondary response to SRBC and the capacity of lymphoid cells to mount a GvH reaction, provide direct evidence to support the conclusion of an activity of this agent on the immune system.

Moreover the data obtained in the L1210 Ha leukemia system showing that NSC 208828 increases the protective effect of X-rayed

Table 7. Effect of NSC D208828 in Lewis-lung carcinoma-bearing C57B1/6 mice

Exp. group	Primary tumor Wt (g ± S.E.)	Mice with metastases (%)	No. lung metastases ± S.E.	Wt lung metastases (mg ± S.E.)
Control	6.94 ± 0.5	100	16 ± 2	41 ± 6
NSC D208828 25 mg/kg i.p.	7.00 ± 0.3	80	8 ± 1*	19 ± 5*
NSC D208828 50mg/kg i.p.	6.70 ± 0.3	100	7 ± 1*	13 ± 3*
NSC D208828 100mg/kg i.p.	7.09 ± 0.6	90	6 ± 1†	13 ± 2†
NSC D208828 150mg/kg i.p.	6.01 ± 0.5	50*	3 ± 1‡	5 ± 1‡
Levamisole 3mg/kg i.p.	6.88 ± 0.6	100	17 ± 2	37 ± 5

*$P < 0.05$ vs control or Levamisole.
†$P < 0.01$ vs control or Levamisole.
‡$P < 0.001$ vs control or Levamisole.
2×10^5 3LL cells were transplanted i.m. in C57B1/6 mice on day 0. Evaluation of primary tumor weight and metastases was performed on day 23. Drugs were administered on day 1.

leukemia cells towards subsequent live tumor challenges, further supports the conclusion that the antitumoral activity of this drug is attributable to its immunostimulatory capacity.

It may additionally be noted that the compound was not cytotoxic *in vitro* for L1210 Ha cells at a concentration of 200 μg/ml (unpublished data). The possibility of inducing allogeneic tumor enhancement by pretreatments with active doses of this agent parallels previous demonstrations of a similar effect with other well known immunomodulators, such as *C. parvum* [14], BCG [15], Levamisole [16] and Pyran copolymer [17]. Since the mechanism(s) responsible for this effect of NSC 208828 have not been investigated, the possibility of a production of blocking factors as seen for tumor enhancement after BCG or Levamisole [16, 18] is just one of the possible hypotheses which can be advanced.

In regard to immunocyte subpopulation(s) stimulated by this drug, in view of its activity on the T-B dependent anti-SRBC response and the absence of detectable effects when the T-independent S III antigen [19] was employed, it would appear that T-lymphocytes are among the targets for this compound. This tentative conclusion is supported by our finding of a stimulation of GvH, a reactivity known to be dependent on T cells for its expression [20]. The relative importance of an increase in the functional capacity of these cells and of an expansion in their numbers is still to be determined. We have in fact found that total spleen cellularity was approximately doubled 7 days after the injection of 100–150 mg/kg of NSC 208828 in mice (unpublished data), an observation which may parallel the finding of an increase in circulating T lymphocyte numbers in normal rats [13].

In the conditions employed, phagocytes did not appear to be significantly influenced by clearly immunostimulatory doses of this chemical as indicated by the absence of changes in the rates of colloidal carbon clearance and in the capacity of purified peritoneal macrophages to non specifically inhibit tumor cell growth. Also unchanged by this drug was the activity of K cells, i.e. of the elements mediating antibody-dependent cellular cytotoxicity, a mechanism currently credited with an important role in tumor control [21, 22], although the precise nature of its effector cell(s) is still matter of debate. In this respect, this agent appears similar to Levamisole a chemically related drug whose capacity to restore to normal levels depressed immune reactivities has been the object of much recent interest [1, 23, 24] and which has been shown to be inactive on K cells in the same system [1]. Levamisole is also believed to exert its effects mainly through T lymphocytes [1, 6] although activites on macrophage-monocytes and polymorphonuclears have also been described [1, 23]. A more definite comparative evaluation of these two chemicals must wait however further studies.

In these experimental conditions the active dose range was generally comprised between 50 and 150 mg/kg and indications were obtained

for best effectiveness at the latter dose level and for a disappearance of activity at still higher dosages, at least in the anti-SRBC system. If confirmed in a larger number of conditions, the latter finding would be an analogy to what seen with other immunomodulators for which the treatment conditions as regards, for instance, dose and timing of injection in respect to the stimulus, can often be critical. It is clear, therefore, that the therapeutically most effective conditions of treatment with this compound have still to be more firmly established through a more systematic analysis in a wider series of experimental models. In conclusion thus, although NSC 208828 has been shown to possess a number of interesting properties, (e.g. potent immunostimulatory activity after single treatments in both neoplastic and non neoplastic systems, capacity to synergize with chemotherapy and effectiveness after oral administration [13] a wide margin of safety between active and toxic doses), further experimentation is necessary before its therapeutic potential is fully assessed and its value relative to other immunomodulators established.

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Morphologic Aspects of Experimental Canine Bronchial Carcinogen Exposure*

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Abstract—*Microscopic alterations of canine bronchial epithelium were studied during the course of recurrent localized carcinogen exposure. Varying sequences and administrations of benzo(a)pyrene and/or* N-*methyl*-N-*nitrosourea were applied to* 5 *groups of dogs. After a period of study ranging from* 24 *to* 34 *months, a relatively orderly progression of epithelial alterations was appreciated, regardless of the type of treatment, based upon monthly biopsies of the site of carcinogen administration. The initial lesion was that of hyperplasia of the columnar cells with occasional intra-epithelial gland formation. Basal cell hyperplasia and squamous metaplasia ensued and were frequently coexistent. Atypical epithelial maturation was noted in all dogs, especially in those whose biopsies exhibited squamous metaplasia. Control sites revealed no epithelial abnormalities. These studies define the evolution of apparently pre-neoplastic bronchial epithelial lesions and also establish the dog as an ideal experimental model for studying the sequence of events during induction of bronchial carcinomas.*

INTRODUCTION

THE EXPERIMENTAL induction of pulmonary carcinoma by chemical means has been achieved by several groups of investigators, but the resulting cancers have not been analogous to the very common bronchogenic carcinomas in man. For example, multicentric carcinomas have been induced in the Syrian golden hamster by intratracheal administration of benzo(a)pyrene (BP) [1], *N*-methyl-*N*-nitrosourea (NMU) [2], or 7H-dibenzo(c,g)carbazole [3]. Schreiber and coworkers have induced circumscribed tracheal cancers with NMU in hamsters [4]. In dogs, Staub *et al.* [5] have caused bronchial carcinomas using 9, 10-dimethyl-1, 2 benzanthracene and Nakajima and co-workers [6] used 20-methyl-cholanthrene to induce cancers. These and other animal models do not usually allow for precise localization of carcinogens, and, therefore, for localized tumor production. In addition, the resulting neoplasms do not always allow for serial observations of oncogenesis. Because the tumors induced may not be similar to the human disease, we are working at producing and studying localized bronchial carcinomas in dogs utilizing a method of recurrent localized intrabronchial injections and/or applications of carcinogens [7]. Our microscopic morphologic observations made serially during a period ranging from 24 to 34 months, and following carcinogen exposure, constitute this report.

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MATERIAL AND METHODS

Five groups of dogs were studied (Table 1). In Group I the carcinogen was BP (15 mg/ml) attached to a ferric oxide carrier as suggested by the work of Saffioti *et al.* [1]. For Group II, NMU freshly dissolved in saline at a concentration of 15 mg/ml was used. For Groups III, IV and V, sequential administration of the two carcinogens was employed. In Group III, BP was injected recurrently into the submucosa until squamous metaplasia was induced, and in Groups IV and V, BP was injected for 7 months;

Table 1. Summary of experimental design

	Test animal	No. dogs	Carcinogen	Volume (ml)	Concentration (mg)	Total dose (mg)	Method	Maximum duration (months)
Group I	Mongrel	7	BP-Fe_2O_3	3	15	810	Submucosal injection	32
Group II	Mongrel	8	NMU	3	10–15	810–945	Submucosal injection	28
Group III*	Mongrel	6	BP-Fe_2O_3	3	15	405–1620	Submucosal injection	11
			NMU	0.5	10	240–360	Mucosal application	16
Group IV*	Inbred Beagle	12	BP	0.5	30	315	Submucosal injection	7
			NMU	0.5	10	135	Mucosal application	17
Group V*†	Inbred Beagle	21	BP	0.5	30	315	Submocosal injection	7
			NMU	0.5	10	135	Mucosal application	17

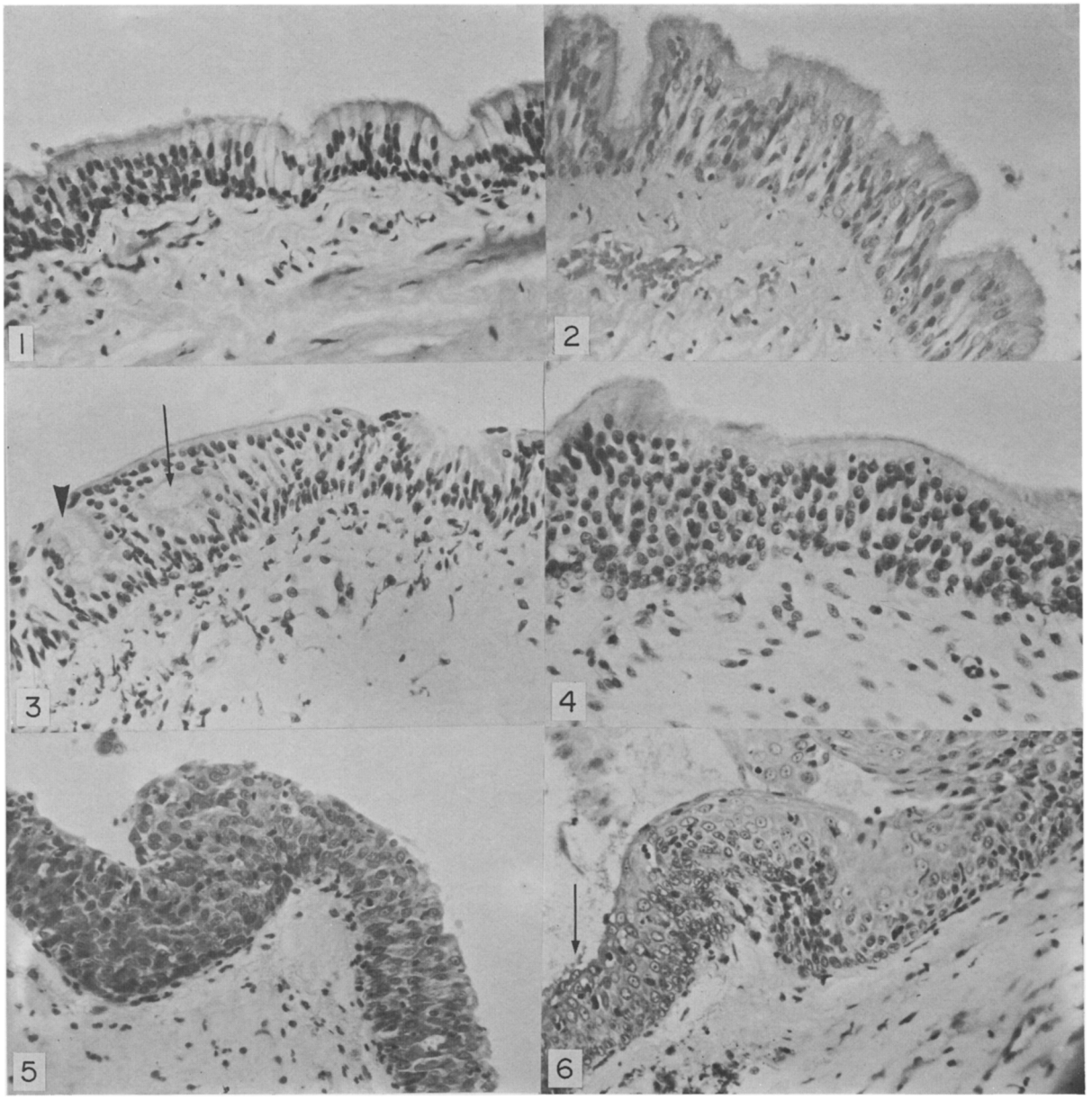
BP—3,4 benzo(a)pyrene.
NMU—*N*-methyl-*N*-nitrosourea.
*BP injections were continued until the appearance of squamous metaplasia at the site of administration; thereafter, NMU was applied locally and BP was discontinued.
† Group V dogs were treated with azathioprine and prednisolone to achieve chronic immunosuppression.

thereafter, the weekly BP injections were discontinued and replaced by topical NMU (5 mg) applications at that same site. This is a modified schedule of that found to be effective in hamsters by Kaufman and Madison [8]. Groups IV and V differed from Group III in that BP was not attached to Fe_2O_3 but injected in a steroid suspension medium. Groups IV and V differed from one another in that Group V dogs were given azathioprine (2.5 mg/kg) and prednisolone (1.0 mg/kg) daily in order to study the effects of immunosuppression on carcinogenesis. Serial monitoring of this regimen was performed after 2, 5 and 9 months of treatment. Antibody responses to bovine and rat red blood cells and chicken gamma globulin were determined to measure humoral immunity. Cell mediated immunity was assessed on the basis of split thickness skin allograft survival; a single mongrel dog served as the common donor for all allografts. Delayed cutaneous hypersensitivity responses to rabies vaccine, distemper–hepatitis–leptospirosis vaccine, and 3,5 dinitro chlorobenzene were determined. Dogs in Group IV served as controls [9].

The origin of the right intermediate (accessory) lobe bronchus served as the site of carcinogen administration. BP was injected into the submucosa in all instances and NMU was applied topically except in Group II dogs where it was also injected. Weekly bronchoscopies at the time of carcinogen administration, and monthly biopsies were performed in all animals. Bronchoscopies and biopsies were continued following cessation of carcinogen exposure. The origin of the left lobe bronchus served as the control site where the solvents for the corresponding carcinogens were administered in the same manner as the carcinogens. Biopsies of control sites were taken at the same intervals as those of the experimental site.

Exposure of the bronchial mucosa to carcinogen was accomplished using a modified esophageal varices needle. For the injection procedure, the tip of the needle was shortened to avoid deep penetration of the submucosa or lung parenchyma. For the topical application procedure, a small sponge was attached to the end of the needle through which soluble carcinogen could be quantitatively applied to the mucosal surface. Targetting of these procedures to the desired location was assisted by direct vision bronchoscopy. A more detailed description of instrumentation and techniques has been previously reported [10].

Fragments of tissue (2 mm) were obtained with biopsy forceps through the bronchoscope. The specimens were fixed in 10% neutral



Note: All sections are stained with hematoxylin and eosin. Number of weeks refers to time after onset of carcinogen application.

Fig. 1. *Normal canine bronchial mucociliary epithelium* (×208). (*Group* I, *dog* 375, *biopsy prior to initial carcinogen exposure*).

Fig. 2. *Columnar hyperplasia. The epithelial layer is thickened by several strata of elongated cells. Note cilia at the luminal surface of the superficial cells* (×208). (*Group* II *dog* 464, 8 *weeks*).

Fig. 3. *Columnar hyperplasia. There are small gland formations* (*arrow*) *apparently the result of infolding of the surface epithelium* (*arrowhead*) *within the considerably thickened mucosa.* (×208). (*Group* V, *dog* 16*B*, 7 *weeks*).

Fig. 4. *Basal hyperplasia. The basal cells have proliferated and constitute the majority of the cellular elements of this thickened epithelium. The surface is composed of ciliated cells* (×208). (*Group* IV, *dog* 10, 15 *weeks*).

Fig. 5. *Extension of polygonal basal cells through the entire thickness of the mucous membrane. Note scattered lymphocytes within epithelium* (×208). (*Group* III, *dog* 101, 34 *weeks*).

Fig. 6. *Well defined area of squamous metaplasia immediately adjacent to focus of complete basal hyperplasia* (*arrow*) (×208). (*Group* II, *dog* 468, 24 *weeks*).

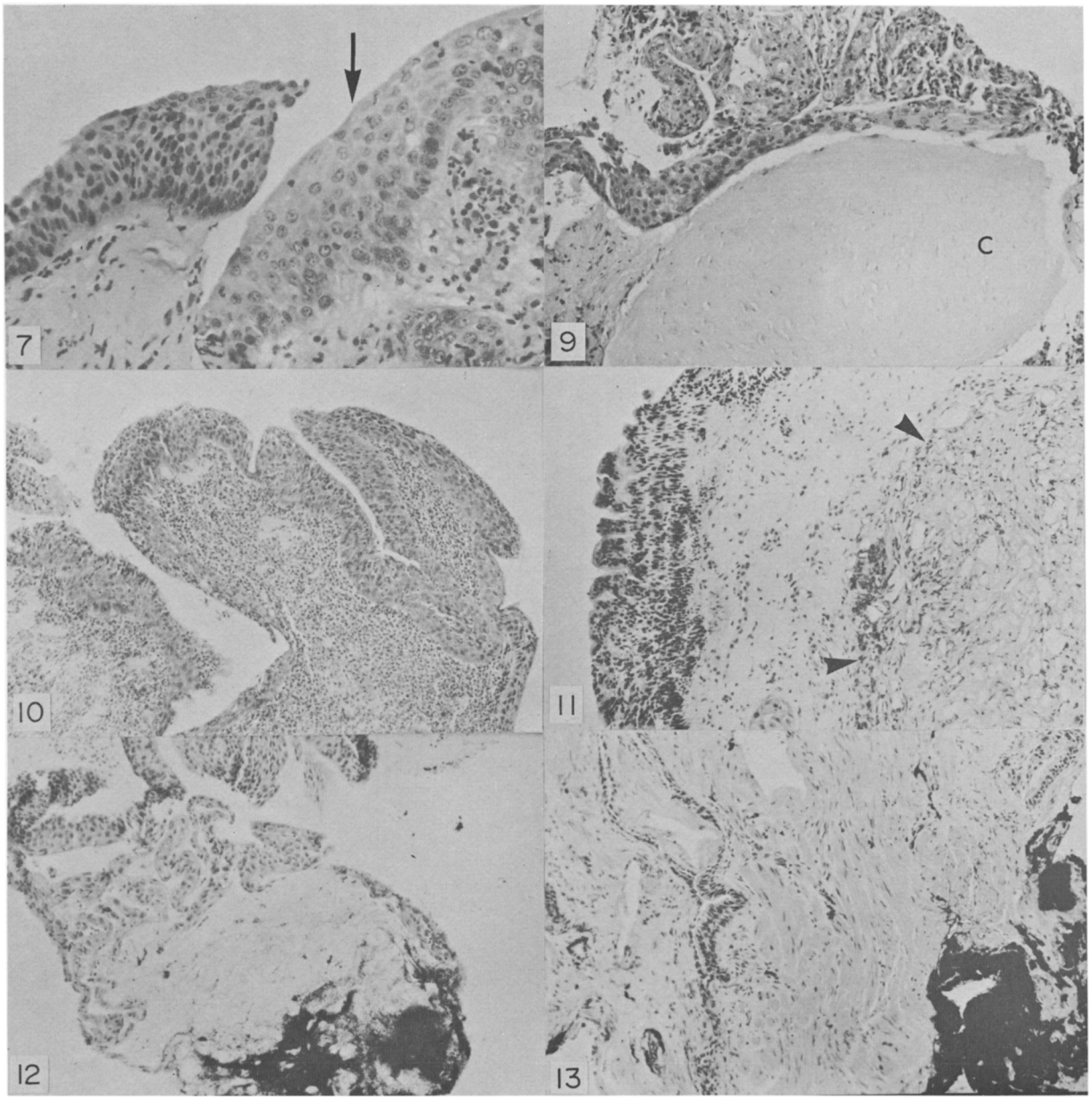


Fig. 7. Regular squamous metaplasia in tissue fragment (arrow) adjacent to one showing dysplastic epithelium characterized by loss of cellular orientation and organization, nuclear hyperchromasia and irregularity (× 208). (Group III, dog 116, 34 weeks).

Fig. 9. Biopsy showing fragmented squamous metaplastic epithelium and portion of a plate of non-viable cartilage (C) (×83). (Group V, dog 12A, 23 weeks).

Fig. 10. Epithelium displaying extensive basal hyperplasia and marked submucosal inflammation; many of the inflammatory cells have extended into the mucous membrane (×83). (Group IV, dog 3, 66 weeks).

Fig. 11. Biopsy with hyperplastic mucosa. Deep in the submucosa is a focus (arrows) of angular clear spaces and a sparse inflammatory reaction (×83). (Group V, dog 19C, 7 weeks).

Fig. 12. Fragmented epithelium with squamous metaplasia. The submucosa is edematous and necrotic and the site of a large deposit of dark ferric oxide pigment (×83). (Group III, dog 116, 73 weeks).

Fig. 13. Biopsy from control site. Note normal epithelium and ferric oxide in submucosa (×83). (Group I, dog 477, 96 weeks).

buffered formalin, dehydrated in alcohols, embedded in paraffin, sectioned at 4–5 μm and stained with hematoxylin and eosin.

In evaluating the microscopic findings, we established these criteria for defining the observed alterations:

Normal—The usual mucociliary pseudostratified respiratory epithelium prevails.

Columnar hyperplasia—The mucosa is thickened, there is an increase in layers of columnar cells.

Basal hyperplasia—The basal or reserve cells, which are small and inconspicuous, become enlarged, polygonal and prominent. Thereafter, they increase in number and add more strata of cells to the mucosa. Although the surface may be composed of ciliated respiratory epithelium, ultimately the basal cells replace the overlying columnar cell element. Mucous cells are usually absent. Other terms for basal cells are germinative [11] or transitional cells [12].

Squamous metaplasia—The mucosa is composed of nonkeratinizing stratified squamous epithelium; keratin is occasionally produced.

Atypia—There is abnormal cellular maturation and/or clusters of cells with an altered nucleocytoplasmic ratio, irregular hyperchromatic nuclei and loss of nuclear polarity. In this lesion, mitotic figures, sometimes of abnormal configuration, are present in various locations in the epithelial layers. Although atypia may be a feature of columnar and basal hyperplasia, it is most frequently associated with squamous metaplasia.

RESULTS

This report is based upon 943 histologic observations from 54 dogs in our study. Neither the various carcinogenic regimens nor the use of adjuvant immunosuppressives, altered the morphogenesis of the abnormalities during the period of this study.

All biopsies prior to the administration of carcinogens were normal (Fig. 1). Microscopic bronchial mucosal and submucosal abnormalities were induced in all dogs. They appeared an average of 7 weeks (range 4–16 weeks) after initial instillation of carcinogens. All dogs had mucosal abnormalities within 3 months.

Hyperplasia of the columnar epithelial cells was observed as the earliest alteration (Fig. 2). In its most pronounced form it was associated with adenomatous transformation of portions of the mucosa and with intramucosal gland formation ("adenomatosis") (Fig. 3). Although columnar hyperplasia was sporadically noted during the course of observation, it was most often present within the first 3–6 months. Proliferation of basal cells (Fig. 4), which was the most frequent change, occurred as the sole mucosal abnormality, or in conjunction with columnar hyperplasia or squamous metaplasia. It was noted as early as one month after carcinogen application and was a constant feature of almost all abnormal biopsies. When affected, the respiratory epithelium was thickened to a variable degree by proliferating basal cells. In the extreme degree, they constituted the entire thickness of the mucous membrane without clearcut surface differentiation (Fig. 5). Squamous metaplasia appeared as early as 4 weeks post-instillation and by 16 weeks nearly all biopsies showed this alteration (Figs. 6 and 7); it was usually non-keratinized. After a variable period of time following the appearance of squamous metaplasia, some degree of dysplastic maturation (atypia) was noted in 40 dogs or 76% of all the animals (Fig. 7). This change has been present on 3 or more biopsies in 20 dogs, or 38% of the animals. However, sequential biopsies have not regularly revealed the atypia shown on previous biopsy.

The evolution of these lesions as a function of time after instillation of carcinogen is presented in Fig. 8.

The mucosal alterations described above represent a summary of the lesions in all dogs. While these changes appear to proceed as described, for each individual animal the progression did not always adhere to the ideal situation and fluctuations from one abnormality to another were frequently noted in successive biopsy specimens. As a result, at any single interval, all biopsies did not show the abnormality expected from the progression proposed here. Hence, the chart in Fig. 8 indicates that at no time did 100% of the biopsies have a specific lesion at any given time. The chart is meant to represent a general summary of the progression

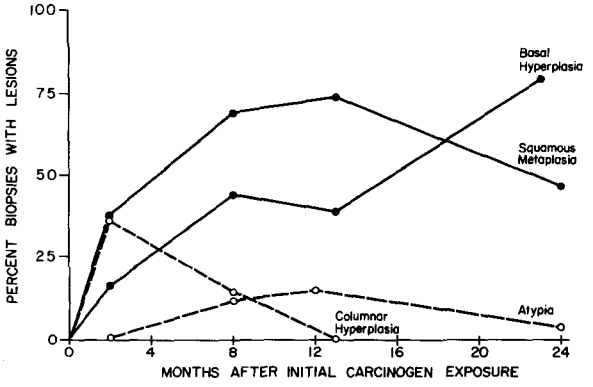


Fig. 8. Evolution of epithelial abnormalities as revealed by serial biopsies. This graph represents a summary of the lesions in all dogs at selected intervals.

of the epithelial changes induced by the carcinogens.

Abnormalities of the submucosa in the biopsies were as varied as those of the mucosa, and their appearance also was dependent upon the site sampled. Submucosal changes were induced by injection of both BP and NMU. The initial lesion was localized necrosis of the collagen, muscularis and cartilaginous plates (Fig. 9). This was associated with leakage of proteins and fibrin, edema and infiltration by variable numbers of polymorphonuclear leukocytes. In many instances, these cells extended into the mucosa (Fig. 10). In sites distant from repeated injections, or upon termination of the treatment, evidence of healing was noted. Fibroblastic proliferation with collagen deposition, proliferation and ingrowth of capillaries, and the presence of macrophages, lymphocytes and plasma cells were observed. In a few biopsy specimens, empty clefts of crystalline shape were noted in the submucosa (Fig. 11). These presumably represented remnants of carcinogen or carrier substance and were associated with extensive areas of necrosis, edema and inflammation. That the submocosal changes resulted from the carcinogens and not from the carriers, or from repetitive biopsies, was attested to in considering the biopsies from Group I. In this group, the presence of necrosis in close proximity to a mass of ferric oxide pigment in the specimen verified that the biopsy was from a site of injection (Fig. 12). There was no constant relationship between the submucosal alterations and those occurring in the overlying epithelium. After the cessation of carcinogen administration, healing by fibrosis was regularly evident in the submucosa. Abnormal fibroblasts or other connective tissue or lymphoid elements were not observed.

The technical and drug toxicity factors which led to the deaths of 31 dogs at various phases of these experiments have been reported in detail [13]. Briefly, 18 of the dogs (Group V) were immunosuppressed and expired either of infectious complications, bone marrow toxicity, and/or generalized debilitation. Four dogs in Group II died of local or systemic effects of excessive NMU; subsequent to these fatalities the dosage was diminished without evident toxicity. Deaths were also due to anesthesia [2] or massive hemorrhage during endoscopy [2]. One severely debilitated dog from Group I was sacrificed and found to have massive intestinal and pulmonary ascariasis. The remaining 4 dogs succumbed to causes which remain uncertain even after postmortem examination. Careful postmortem examination of all these dogs revealed that the spectrum of bronchial mucosal changes, as indicated above, was present in the same animal. Serial sectioning of the region of the orifice of the right intermediate lobe bronchus indicated that the various epithelial lesions could be either adjacent to and admixed with one another or widely separated. The degree or appearance of columnar hyperplasia was minimal or absent in the autopsy specimens, most likely because the deaths usually occurred after the sixth month of the experiment. One of the dogs which died at the time of the first monthly biopsy exhibited extensive columnar hyperplasia with adenomatosis at autopsy. Basal hyperplasia and squamous metaplasia were minimal. In all other dogs, columnar hyperplasia was minimal or absent in the autopsy specimens, most likely because the deaths occurred after the sixth month of the experiment. Squamous metaplasia and atypia were constant findings in all of the autopsy specimens. The metaplastic alterations were adjacent to either normal, or more frequently, hyperplastic epithelium. Additionally, the atypical features affected mainly the squamous epithelium, although dysplastic basal hyperplasia also occurred. Abnormal epithelium and occasionally normal epithelium, covered submucosa displaying the changes described above. However, the extent of fibrosis could be better appreciated in the autopsy specimens; vast areas of scarring responsible for considerable deformity of the bronchial contour were evident. As a result, many sites with severe epithelial abnormalities were apparently inaccessible to the biopsy forceps.

In all cases, biopsies from control sites revealed no mucosal alterations; occasional specimens showed submucosal edema and/or mild inflammation. As alluded to previously, it was readily possible to determine if the tissues sampled represented the location of the control injection because of the presence of Fe_2O_3 deposits (Fig. 13). Approximately 50% of the biopsies in Group I contained collections of red-black ferric oxide pigment which served to indicate a site of injection. Detailed observations on numerous sections of the left lower lobe bronchus taken at all autopsies also revealed an absence of significant mucosal and submucosal alterations.

Morphologic abnormalities associated with systemic manifestations of carcinogen administration, e.g. bone marrow hypoplasia, cellular atypia and/or neoplasia were not evident in any extrabronchial site examined in the dogs that died.

Although the microscopic alterations affecting

the bronchial wall are emphasized here, significant stenosis of the bronchial orifice, leading to complete obstruction in a small number of dogs from all groups, was evident by bronchoscopic examination. This finding extends our previous observations of stenosis affecting bronchi exposed to carcinogen [7, 13].

The results of the immunosuppression regimen indicated that antibody responses were depressed within two months, skin graft rejection was prolonged, and cutaneous responses to the injected antigens were suppressed, as compared to controls (Group IV) [9].

DISCUSSION

With the current clinical and experimental interest in respiratory tract oncogenesis, it is clear that pre-neoplastic changes of the tracheobronchial tree deserve attention [4, 14]. We have defined sequential morphologic alterations induced by recurrent carcinogen applications in the canine bronchus. Our study shows that the use of serial biopsies is a practical method with which to study the effects of substances such as carcinogens on the various bronchial tissues in large animals. Others have shown that chemical carcinogenesis in dog lungs is possible [5, 6], and we, therefore, feel that our dogs will also develop cancers. Our focus currently is upon pre-neoplastic lesions, partly because that is now the stage of our experiments and partly because that is the likely area for clinically significant diagnostic and therapeutic progress [14]. We have described the microscopic evolution of the canine bronchus exposed to non-carcinogenic recurrent trauma and to one or two carcinogens, and have noted that carcinogen-induced lesions progress through a relatively orderly sequence. Hyperplasia of columnar epithelium with or without adenomatosis, proliferation of the basal cells, squamous metaplasia and atypia represent to date the response of canine bronchial epithelium to carcinogen application. These changes seem to persist even after cessation of carcinogen exposure.

The events which occur in the submucosa in response to carcinogen exposure are likewise sequential and progressive. Deposition of chemicals, necrosis, inflammation and macrophage activity are followed by evidence of scarring and chronic inflammation with concomitant distortion of the architecture. Because the carcinogens have been introduced into the submucosa, we realize the possibility that malignancies other than carcinomas, e.g. fibrosarcoma, leiomyosarcoma, etc. may also be produced during the course of this study. Although BP and NMU have been effective in inducing pulmonary carcinomas [1, 2, 15], they have also been utilized in the production of soft tissue malignancies [1, 16]. As demonstrated by Shabad and Pylev [17], fibrosarcomas, angiosarcomas and reticulosarcomas may be induced in the course of bronchial epithelial carcinogenesis.

The effects of carcinogen injection on the bronchial submucosa were similar for both BP and NMU in our animals. This observation is not surprising in light of the work of Hoosen *et al.* [18] who evaluated the early reaction of subcutaneous tissue to repeated injections of different carcinogens. They found that these substances cause destruction of collagen, inhibition of cell multiplication and induction of cytomegaly. It appears that the bronchial submucosal effects observed by us are predominantly those of carcinogen exposure (i.e. destruction of collagen), although reparative and proliferative changes also occurred.

Our study has revealed that there is no basic difference in the evolution of lesions induced solely or primarily with BP as compared to NMU. Although our initial data [7] suggested that the epithelial changes seemed to appear more promptly with BP than with NMU, our current results do not support that impression. The use of a larger number of animals has permitted us to note the wider temporal variation in response to carcinogen application; hence, what appeared initially to be significant differences in rate of development of cellular changes actually represented individual variation among the animals.

Epithelial alterations similar to those described in this study on dogs have been noted in rodents whose respiratory tracts were exposed to various carcinogens. Harris *et al.* [15] studying the Syrian golden hamster treated with BP, described the earliest abnormality as epithelial hyperplasia which corresponded to the basal hyperplasia in our group of animals. Similarly, in animals exposed to NMU, basal hyperplasia of the tracheobronchial epithelium was consistently produced [15]. This type of change was considered to be the earliest abnormality. In contrast is our finding of a rather pronounced hyperplasia of cells that we identified to be of columnar origin. Following columnar hyperplasia, we observed basal hyperplasia usually in association with squamous metaplasia. Basal hyperplasia, on the other hand, was less commonly noted in conjunction with columnar hyperplasia. Columnar hyperplasia was instead frequently associated with "adenomatous" metaplasia or gland formation within the thickened mucosa, a lesion that has not been previously

emphasized either in experimental animals or in humans. Shabad and Pylev [17] described similar hyperplastic lesions of columnar epithelium in the bronchi of rats treated with intratracheal instillation of 7,12-dimethylbenz(a)anthracene. However, the proliferation of cells was frequently of a papillary nature and consisted of several layers of columnar or cuboidal cells extending into the bronchial lumen.

The fact that similar or progressive mucosal alterations did not appear on every biopsy, and that the sequence of events that we have described in detail was not necessarily reflected in every specimen sampled was initially disturbing to us. For example, the absence of cellular atypia on a biopsy after it was noted on a previous one was of concern. We felt that the lesions produced were "regressing" despite continued gross bronchoscopic changes, and in some cases the continued application of carcinogens. Our observations on autopsy tissue helped to resolve the questions posed by these observations. Postmortem examinations, with multiple sections of injection sites, on the dogs which were studied for greater than 3 months, revealed the frequent coexistence of normal and abnormal tissue. In fact, the entire spectrum of mucosal abnormalities occurred within a radius of 1 cm of the application sites. In addition, the degree of deformity and stenosis of the orifice of the right accessory lobe bronchus resulting from the submucosal alterations made it virtually impossible to sample many of the more affected areas during the bronchoscopic procedure. It is for these reasons that we feel that the inconstant biopsy findings in individual dogs do not represent a variability in the biologic process, but far more likely represent sampling errors. The importance of these observations in relation to human bronchial lesions cannot be overemphasized. In the face of convincing gross morphologic changes, a single negative bronchoscopic biopsy should not be taken to indicate the absence of significant disease. Multiple biopsy specimens are necessary.

The question of reversibility following carcinogen exposure is of basic importance. Griesemer *et al.* [12] noted that upon cessation of acute exposure of transplanted rat trachea to 7,12-dimethyl(a)anthracene or to BP, most of the hyperplastic-metaplastic lesions initially produced were replaced by normal or near-normal mucociliary epithelium. These investigators felt that this was evidence of regression of the changes. They also noted the appearance of pleomorphic-atrophic epithelium, the nature and significance of which was uncertain. Whether these abnormal cells are those which will ultimately be precursors of a malignant process, should it occur, is not clear. Our observations cannot yet contradict the conclusions of Griesemer *et al.* [12] conclusively, but the trend of our results is in that direction. So far, the bronchial epithelial lesions we have induced in dogs have not regressed even after the carcinogens were stopped.

On the basis of our results to date and those of others utilizing the same carcinogens in the respiratory tract of rodents [1, 2, 15], we expect that the mucosal alterations discussed here represent precursors of malignant tumors. Saffiotti *et al.* [1], Harris and others [15, 19] have shown that the progression of normal to malignant bronchial epithelium proceeds through a relatively orderly sequence of events, all of which have been achieved by us, save for the appearance of the final stage of cancer. In rodents, squamous and other carcinomas have developed generally within one year of carcinogen exposure. However, in the few models in which localized carcinomas were induced in dogs, the tumors did not appear as rapidly. The cancers resulting from weekly bronchial applications of 20-methylcholanthrene in dogs by Nakajima *et al.* [6] appeared from 2 yr 5 months to 3 yr 10 months after onset of the experiment. In a somewhat related setting, Howard [20] utilizing $^{239}PO_2$ particle inhalation carcinogenesis in dogs, induced lung cancers in beagles 38–110 months after initial exposure. It seems that in the canine model a considerably longer period of time is necessary for development of pulmonary malignancies than in other species exposed to similar conditions. We believe that the lack of tumorogenesis in our group of animals to date reflects this species variation. Alternatively, it may also be indicative that our carcinogen regimen in dogs is less rapid than that employed by others [5, 6]. Nevertheless, it is expected that carcinomas derived from bronchial mucosa will eventuate.

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Interaction of Adriamycin and Radiation in Combined Treatment on Mouse L-Cells

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Abstract—*Using an established cell line, we have studied the survival properties after combined action of adriamycin and radiation. The results show that subtoxic concentration of adriamycin sensitizes the cells towards irradiation. This effect can be observed on the modified survival curve, showing a decrease in the extrapolation number. The cell survival after exposure to simultaneous action of both agents was essentially lower as compared with superposed survival after single treatment. If treatments were applied separately, cell survival increased. This equalled the superposed survival when the time interval between treatments was longer than* 3 hr. *The observed synergistic effect depended upon the concentration of acriamycin.*

INTRODUCTION

ADRIAMYCIN is a relatively new antibiotic of the anthracycline group which exhibits antitumor activity with a high therapeutic toxicity ratio [1, 2]. This antibiotic by binding to nucleic acids inhibits cellular DNA and RNA synthesis [3]. Although early S-phase cells are the most sensitive, progression delay was observed in all phases of the cell cycle [4]. In contrast to some cell cycle dependent drugs, adriamycin may have a killing effect on dividing and non-dividing cells [5]. Thereby, it has been suggested that this agent may be an effective drug on slow growing tumors.

Recently some authors have indicated that adriamycin also increases the radiosensitivity of tumor [6–8] and that combined therapy may prove to be useful. The results obtained with some *in vitro* studies [9] indicated that adriamycin, although affecting the radiation survival curve, did not inhibit split-dose repair. Investigations reported in this paper provide further data on the synergistic effect of adriamycin and irradiation.

MATERIAL AND METHODS

Mouse L-cells, clone 929-E, were cultivated on plastic Petri dishes in Dulbecco-modified minimal essential medium containing streptomycin and penicillin supplemented with 5% fetal calf serum (Gibco).

Cell survival was estimated by the clone forming ability of single cells after 12 days, according to the method of Puck and Marcus [10]. In order to gain the same number of clones in irradiated, treated and control dishes, the appropriate number of cells was inoculated. The control suspension was diluted to yield about 100 clones per dish. After plating, dishes were incubated at 37°C for 20 hr. At the end of the incubation period, the average cell multiplicity was about 1.2. For the data shown in Fig. 1, asynchronous cells were obtained by trypsinizing logarithmically growing cultures. Cell cultures were kept in 5% CO_2 in air and the temperature throughout was 37°C. Adriamycin was obtained from Farmitalia-Milano, Italy.

A stock solution of adriamycin was prepared in the medium immediately before each experiment. Certain volumes of this solution were added to each Petri dish so that the expected final concentrations were obtained. The drug was washed from the cultures with prewarmed medium after an indicated time and then incubated further for 12 days with 5 ml of complete medium without drug.

The irradiation was performed by 70 kVp X-ray machine (Phillips) with a dose rate of 100 R/min. In the experiments designed to investigate the synergism of adriamycin and irradiation the samples were exposed to 400 rad, and treated with certain concentrations of adriamycin for 3 hr.

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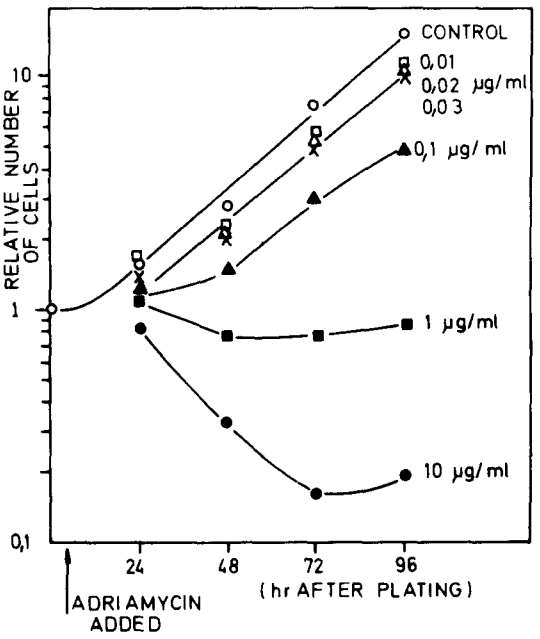

Fig. 1. Effect of adriamycin on cell division. Adriamycin was added 5 hr after plating. Open circles—untreated control, open squares—0.01 μg/ml, open triangles—0.02 μg/ml, crosses—0.03 μg/ml, solid triangles—0.1 μg/ml, solid squares—1 μg/ml, solid circle—10 μg/ml.

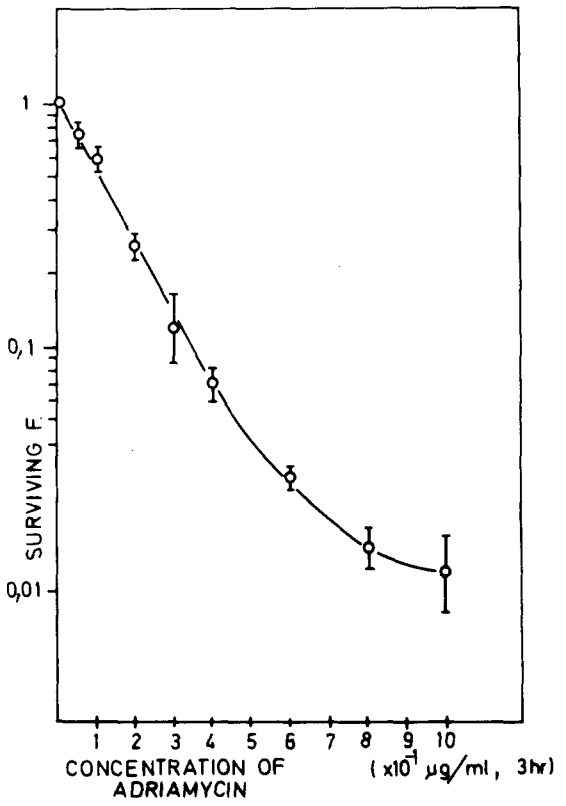

Fig. 2. Cell survival after treatment with adriamycin for 3 hr.

RESULTS

1. *Effects of adriamycin on cell division*

Asynchronous exponentially growing cells were exposed to various concentrations of adriamycin continuously. Nontreated control cells grew exponentially with doubling time 20 ± 1 hr. At concentrations 0.01–0.03 μg/ml cell division was delayed, however at higher concentrations the growth rate seemed to change significantly (Fig. 1).

2. *Effect of adriamycin on cell survival*

Exponentially growing cells were exposed to various concentrations of adriamycin for 3 hr. Survival rates after this treatment are shown on Fig. 2. The biphasic curve obtained is similar to the survival curve of some other antitumour-antibiotics (e.g. bleomycin) [11].

3. *Effect of nontoxic adriamycin concentration on cell survival after irradiation*

Before irradiation with various doses of 70 kVp X-rays, the cells were exposed to 0.02 μg/ml adriamycin for 3 hr. This concentration of adriamycin is nontoxic with a 100% survival rate (see Fig. 2). The survival curve became completely exponential. It is obvious that adriamycin caused a lower survival rate at the same dose exposition due to the decrease of extrapolation number.

4. *Synergism of adriamycin and irradiation*

Samples were exposed to 400 rad 1, 2, 3 and 4 hr before adriamycin (0.02, 0.2 and 1 μg/ml for 3 hr) was added. Next series of samples were irradiated immediately 0.5, 1, 2 and 3 hr after drug was added. Finally, the remaining samples were irradiated by the same dose 1, 2, 3 etc. hr after the removal of the drug. The control group of samples was treated simultaneously in the following way: the first samples were treated with adriamycin only, the second group was only irradiated and the third group of samples remained untreated control. Samples which were only treated with adriamycin gave the survival A. The irradiated control samples gave the survival B. Results are shown on Fig. 4. The zone inside the dotted lines represents the corresponding superposition level of survival obtained by multiplying A × B (see e.g. [12]) and accounting for s.e. of this product. All results have been calculated assuming survival 1 for untreated and unirradiated control. The data show that the highest killing effect was obtained when the radiation was given at the same time and up to 1 hr after adriamycin.

DISCUSSION

Results show that subtoxic concentration of adriamycin sensitized the cells towards irra-

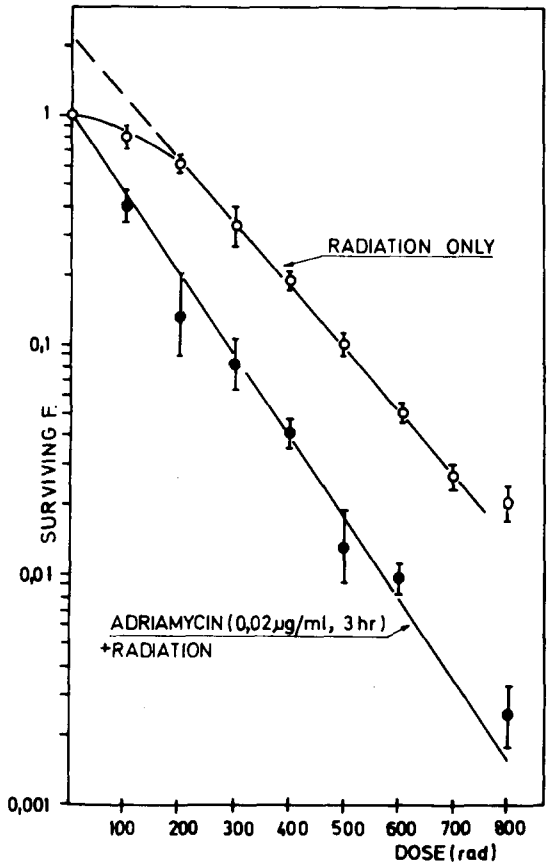

Fig. 3. Cell survival after irradiation. Open circles—only irradiated, closed circles—irradiated after treatment with 0.02 μg/ml *adriamycin for* 3 hr.

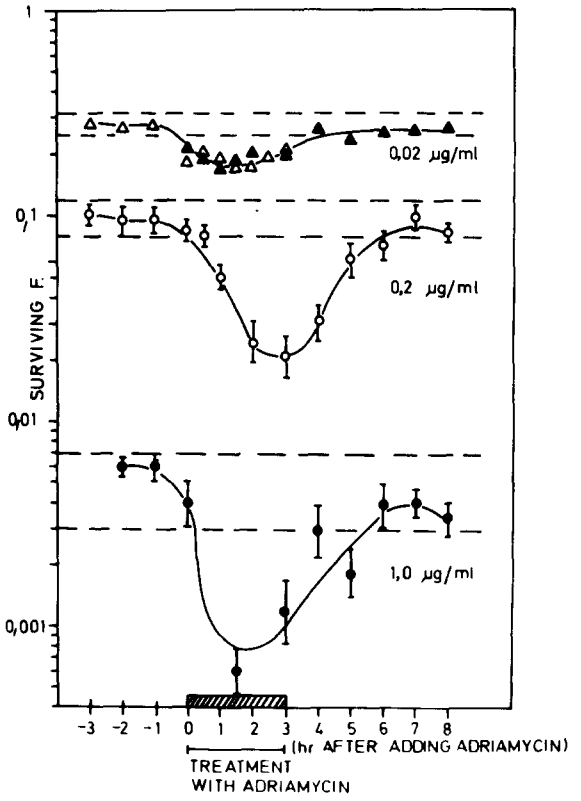

Fig. 4. Combined action of 400 rad *ionizing irradiation and adriamycin treatment for* 3 hr. *Open and solid triangles*—400 rad + 0.02 μg/ml, *open circles*—400 rad + 0.2 μg/ml, *solid circles*—400 rad + 1 μg/ml. *Dotted lines define the corresponding region of superposition.*

diation (see Fig. 3). This effect could be related to the modification of the survival curve, followed by decrease in the extrapolation number. An intensive synergistic effect was observed when a simultaneous action of adriamycin and irradiation occurred (Fig. 4). Synergistic effect increased progressively with time achieving its maximum value 1 hr after adriamycin was added and continued to last 1 hr after drug was removed. A high synergistic ratio (lowest survival/superposed survival) 1:7 was obtained by increasing the adriamycin concentration. When, however, adriamycin treatment and irradiation were separated by a time interval longer than 3 hr, only the superposing effect was observed.

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Malignancy and Prognosis Evaluated by an Embryonic System

Grading of Breast Tumours*

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Abstract—*An embryological system for grading tumours with regard to their malignancy was described by the authors in earlier publications. This system is based on cellular responses induced by tumour cells on implantation into chick embryo blastoderms. In this communication the ectodermal cellular responses induced by human breast tumour cells are described. Carcinomas of the breast induce intense cellular responses, but those induced by the nonmalignant cells of cystic hyperplasia are feeble. The breast tumours and hyperplastic tissue have been embryologically graded, and an apparent correlation between embryological grade and histology is indicated.*

INTRODUCTION

TUMOUR tissues implanted into chick embryo blastoderms induce cellular responses from the embryonic tissues [1–3]. These responses appear to have histogenetic specificity. Tumours of endodermal origin, for example, most actively induce characteristic proliferative responses from the embryonic endoderm [4] but astrocytic tumours which are neuroectodermal in origin induce cellular responses from the embryonic ectoderm [5]. We describe here the induction of an ectodermal cellular response in embryos by human breast tumours. The cellular responses also appear to be related to the state of progression of the tumour and may be scored to provide a numerical grading which correlates with the degree of tumour malignancy [6]. In a series of human astrocytic tumours we have used embryological grading to determine malignancy and have observed that the grading correlates well with prognosis [5, 7]. In this communication we also examine the embryological grading of some human breast tumours in relation to their histology.

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MATERIAL AND METHODS

The breast tumour cells tested were obtained from tissue cultures of breast biopsy specimens. They were donated by Dr. P. A. Riley and were derived from early stages of tissue cultures maintained as described by Riley *et al.* [8]. The cells were detached from culture flasks using a rubber "policeman", washed with Pannett–Compton saline, pelleted by centrifugation and implanted into 16–18 hr old chicken blastoderms [9, 10]. More than 15 implants were made of each tumour. The embryos with the implanted tumour tissue were incubated for a further 18 hr, when they were fixed and examined histologically. The cellular responses were scored and the tumours embryologically graded as described previously [6].

RESULTS AND DISCUSSION

1. *The ectodermal response*

The tumours were found to induce intense responses from the ectodermal cell layer of the blastoderm (Table 1, Figs. 1–6). The structures induced in the ectoderm bear much similarity to neural tissue in their characteristic palisaded cellular arrangement (Fig. 1). In some cases the induced structures were found to have undergone distinct morphogenesis into neural tube-like structures (Figs. 2 and 3). The tumour cells which produced the response illustrated in Fig. 3 may be seen in Fig. 4. The structure illustrated in Fig. 5

Table 1. Cellular response of chick embryo blastoderms to breast tumour implants

Patient	Host mesodermal response (%)	Ectodermal response (%)	Ectodermal response degree	Endodermal response (%)	Endodermal response degree	Morphogenetic displacement (%)
MCP	11.0	31.0	+	14.0	+	23.0
MCF	10.0	67.0	2+	50.0	+	27.0
MCD	0.0	69.0	2+	29.0	+	0.0
AME	13.0	100.0	2+	66.0	2+	14.0
BAS	42.0	100.0	2+	73.0	+	13.0
HOR	43.0	100.0	3+	70.0	2+	15.0
*Non-malignant**						
BAI (Cystic hyperplasia)	0.0	20.0	−	0.0	±	4.0
222 (meningioma)	80.0	66.0	+	0.0	−	0.0

*A meningioma has been included here only to provide comparison (and obviously is not a part of this series).
The tumors are graded according to the following method of scoring: Host mesodermal response 75–100% 0 pt, 50–74% 1 pt, 25–49% 2 pts, 0–24% 3 pts. Ectodermal and endodermal response (frequency) 75–100% 3 pts, 50–74% 2 pts, 25–49% 1 pt, 0–24% 0 pt. Ectodermal and endodermal response (degree) + 1pt, 2+ 2pts, 3+ 3pts. Morphogenetic displacement 75–100% 3 pts, 50–74% 2 pts, 30–49% 1 pt, 0–29% 0pt.

is reminiscent of a secondary embryonic axis induced by embryonic organisers in similar intrablastodermal implantation experiments.

2. *Nature of the histogenetic response*

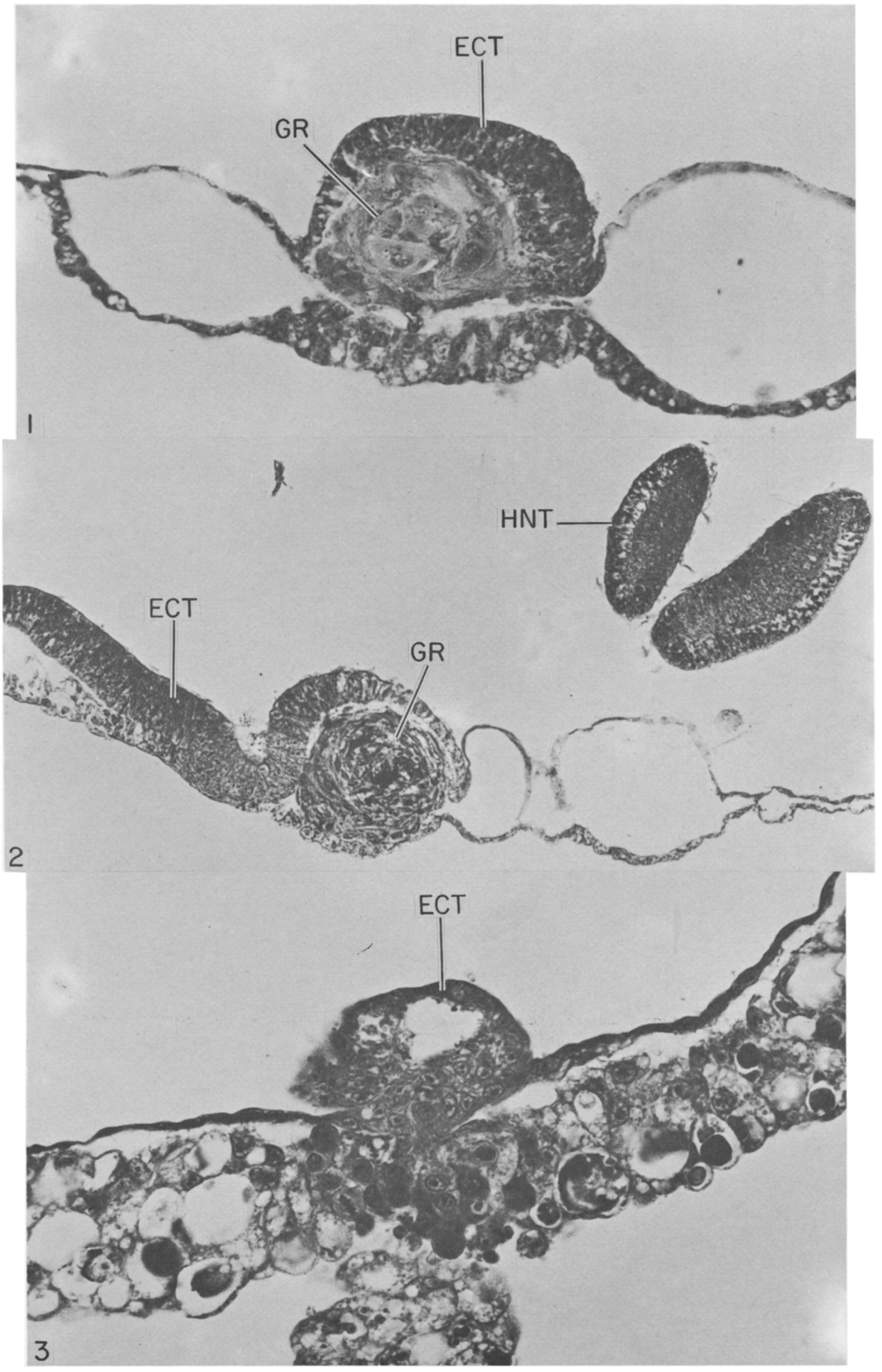
There is now ample evidence which suggests that the induction of histogenetic responses in embryos may be a characteristic of neoplasms (Table 2). Human astrocytomas and breast tumours are the only neoplasms which have induced ectodermal response, in a series of tumours of various histological origin that we have investigated to date. Palayoor and Batra [2] observed that mammary carcinomas of C3H mice were able to induce neuroid reaction, using the chick embryonic system. Both astrocytomas and breast tumours are of ectodermal origin. Of the mesodermal tumours, Yoshida ascites sarcoma and leukemic bone marrow cells have been found able to induce a low level of proliferation of the ectoderm. On the other hand, tumours of endodermal origin appear to induce most actively a response in the embryonic endoderm, although in the present experiments carcinomas of the breast have been able to induce responses in the endoderm (Table 1).

One can only speculate as to the mechanisms by which these histogenetic responses may have been induced, from analogous tissue interactions in embryogenesis. The induction of differentiation of neural tissue by implantation of neural tissue [12, 13], the induction of limb bud by implanted limb buds [14, 15] are well known instances of "homogenetic inductions". The homogenetic inductions are in fact very closely associated with the state of differentiation of the implanted tissue. Competent embryonic ectoderm is capable of inducing neural tissue differentiation only when it has itself been induced to differentiate in that direction. The histogenetic response produced by astrocytomas and breast tumours may reflect the acquisition of embryonic characteristics by dedifferentiation accompanied by an acquisition of the ability to synthesise substances which can alter the path of differentiation of the ectoderm. Inappropriate synthesis of substances is not unusual in neoplasms. Tumours are known to be accompanied by paraneoplastic syndromes of endocrinopathy. Not only has ectopic production of hormones by some neoplasms been abundantly documented [16], but it has also become apparent that this ability may be closely related to the state of differentiation of the neoplasm [17].

3. *The embryological grading of breast tumour malignancy*

The tumours were found to belong to various embryological grades between 9 and 15 indicating increasing malignancy in that order. The histology of these tumours appears to confirm the differences in their malignancy predicted by the embryological grading (Table 3).

In comparison with the responses induced by the malignant carcinomas, those produced by nonmalignant hyperplastic tissue of the breast seem innocuous (Table 1). One such case examined was given embryological grade 4, as also was a nonmalignant meningioma. This is much higher than normal liver cells of rat which elicited a grade 1 response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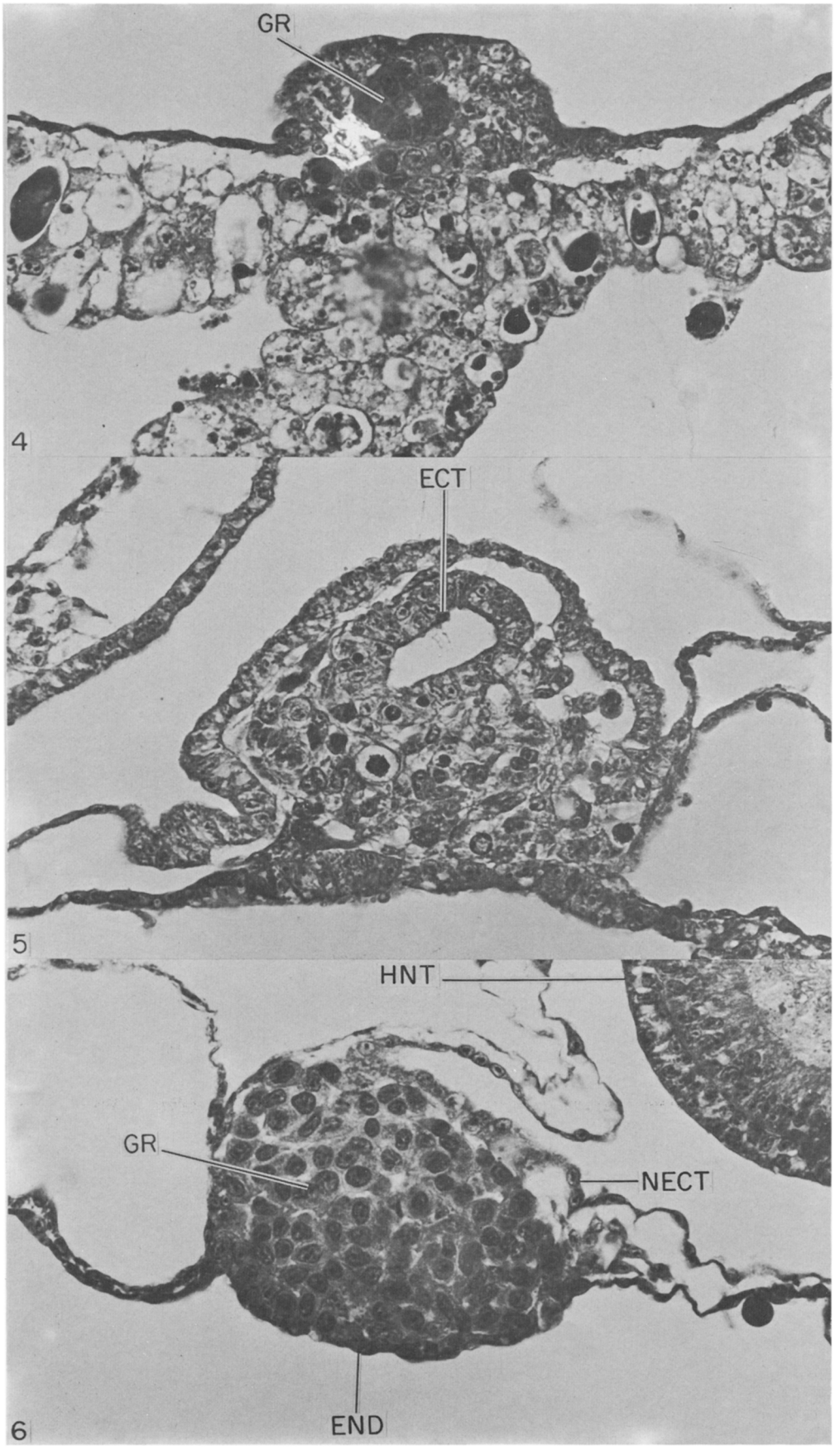

Figs. 1–6. *Ectodermal response induced by human breast tumours. Moderate response* (2+) *in Fig.* 1: *strong response* (3+) *in Figs.* 2, 3 *and* 5 *where the induced structures have undergone morphogenesis into neural tube-like structures. For comparison Fig.* 6 *illustrates absence of response in ectoderm* (*NECT*) *by a breast tumour implant. GR tumour cells, ECT the ectodermal response, END endodermal layer, HNT part of host embryo. GR in Fig.* 4 *are the tumour cells which produced the ectodermal response illustrated in Fig.* 3.

in this system [6]. The embryonic system was able to distinguish between the experimentally induced minimum-deviation tumours of rat, which have a wide spectrum of differentiation and malignancy. Even the very slow growing minimum-deviation rat hepatoma 9633, which is a well-differentiated tumour and which has no metastatic ability, could be distinguished from normal liver implants from which the tumour may be said to differ in the main in its slightly more rapid rate of growth. This discriminatory ability of our system is presumably reflected in the production of low grade responses by cystic hyperplastic tissue of the breast and nonmalignant meningioma. In contrast, the more malignant astrocytic tumours [5] and mammary carcinomas produced much more intense responses from the embryonic tissues, and generally belonged to much higher embryological grades.

It appears therefore that the embryonic system possesses a fair degree of sensitivity

Table 2. Histogenetic specificities of embryonic responses

Tumour	Ectodermal response	Endodermal response	Reference
Ectodermal tumours			
Human astrocytoma	+	±	5
Human carcinoma of the breast	+	+	(This work)
C3G(J) mice mammary adenocarcinoma	+	−	2
Mesodermal tumours			
HeLa (Human carcinoma of the cervix)	−	−	1
Yoshida ascites sarcoma (rat)	±	−	
Landschutz ascites sarcoma (murine)	−	−	
Benzpyrene-induced sarcoma (rat)	−	−	
Leukemic bone marrow	±	−	11
Endodermal tumours			
Minimum-deviation hepatoma (rat)	−	+	4, 5
Dimethylaminoazobenzene-induced hepatoma (rat)	−	+	

Table 3. Embryological grades of some human breast tumours and their histology

Tumour	Embryological grade*	Histology†
BAI	4	Cystic hyperplasia with some areas of epitheliosis
MCP	9	Trabecular amd tubular mucus-secreting adenocarcinoma with a light infiltrate of lymphocytes, plasma cells and histiocytes in the fibrous stroma
MCF	11	Scirrhous carcinoma with slight lymphocytic reaction, chronic inflammation but no growth in 7 lymph nodes
MCD	13	Poorly differentiated adenocarcinoma with poor lymphocyte response. Lymph nodes free of tumour
BAS	13	Poorly differentiated adenocarcinoma with lymphatic infiltration. Lymph nodes free of tumour
AME	14	Invasive carcinoma. Tumour with cellular area of anastomosing trabeculae of spheroidal cells with fibrous reaction of scirrhous adenocarcinoma. No lymphocytic response
HOR	15	Poorly differentiated finely trabecular carcinoma incompletely contained by fibrous reaction with lymphatic permeation.

*Determined as prescribed previously [6] (see footnote to Table 1).
†Histological data was obtained from the Department of Morbid Anatomy, University College Hospital Medical School.

which enables it to respond to discrete differences in the normality and of degree of malignancy of the implanted tissues.

Although the embryological grades of breast tumours investigated here appeared to correlate well with the histology, no follow-up studies have been made. This is because we recognise that selective pressures may operate in the tissue culture phase, and therefore the embryological grades obtained with cells grown in culture may not reflect prognosis accurately. We propose to investigate by direct implantation, i.e. without interposing a tissue culture phase, a large series of breast tumours, in conjunction with a follow-up study over a period of 5 yr.

We consider that the embryological system of grading may find its most rewarding application in the management of breast cancer where the form of treatment is closely linked with the clinical stage of the disease and, as Mansfield [18] has pointed out, one is posed with the dilemma of deciding whether to follow conservative forms of treatment or to adopt more radical procedures. It is hoped that our proposed future studies using the embryological malignancy grading system will be helpful in this respect.

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Soluble Factors Produced by Normal or Tumor Cells, Inhibiting the Uptake of 5-Iodo-2′-deoxy-uridine by Cancer Cells *In Vitro**

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Abstract—*The uptake of* ^{125}*I-5-iodo-2′-deoxy-uridine* (^{125}*IUdR) by lymphoma cells is inhibited in lethally irradiated hosts incompatible for* Hh (*hemopoietic-histocompatibility*) *loci. In the course of attempts to reproduce this phenomenon* in vitro, *a soluble factor* (*uptake inhibiting factor, or UIF*) *was found in the supernatant of cultured* (16 hr, 37°C) *spleen cells. UIF was produced by non-stimulated splenocytes of various strains of conventional and athymic* (*nude*) *mice and rats and by various tumor lines as well. UIF was found to be non-strain, non-species and non-tissue specific, since it was capable of inhibiting the* ^{125}*IUdR uptake of mouse, hamster and human tumor cells of various tissue origins. Lethal irradiation, silica or IDF* (*immunodepressive factor*) *treatment of donor mice, or irradiation or treatment with actinomycin D of spleen cells* in vitro, *did not prevent UIF production by such cells. On the other hand* 5-*fluoro-2′-deoxy-uridine* (*FUdR*) *reduced UIF production by* 50%. *UIF was found to be relatively resistant to heat and proteolytic enzymes* in vitro. *In addition UIF was capable of cytostatic or cytotoxic effects on tumor cells in tissue culture. UIF-like activity was found also in the plasma of tumor bearing mice. The biological role of UIF seems to be uncertain. Nevertheless it could interfere markedly with* in vitro *assays involving cell proliferation monitored by DNA-precursor uptake.*

INTRODUCTION

NORMAL hematopoietic or lymphomatous cells do not proliferate in the spleen of lethally irradiated recipient mice incompatible for the *Hh* locus [1–8]. The uptake of 5-iodo-deoxy-uridine ^{125}I (^{125}IUdR), a non-physiological precursor incorporated into DNA in place of thymidine [9], has been used to monitor cell growth or proliferation inhibition in spleen, liver and lung of irradiated recipients [5–8].

Attempts were made in our laboratory to reproduce *in vitro* ^{125}IUdR uptake inhibition of lymphoma cells by coculturing them with spleen cells obtained from lethally irradiated, *Hh*-incompatible donor mice. The data of several experiments indicated that non-specific inhibition of ^{125}IUdR uptake occurred in lymphoma cells when cocultured *in vitro* with either syngeneic or allogeneic splenocytes.

Soluble factor(s) capable of inhibiting the ^{125}IUdR uptake by cancer cells was found in the supernatant of cultured spleen or tumor cells. The factor (hereafter called UIF, or uptake inhibiting factor) reduced or abolished not only ^{125}IUdR uptake but also tumor cell proliferation *in vitro*. UIF was found to be relatively resistant to heating and to treatment with proteolytic enzymes *in vitro*. It was produced in fairly constant amounts by splenocytes of various mouse strains, including nude (athymic) mice, whereas UIF production by cancer cells varied greatly with the type of tumor line used. In addition UIF-like activity was found in the plasma of tumor bearing mice.

MATERIAL AND METHODS

Animals

Inbred mice 2–4 months old of both sexes of C57Bl/6Cr(*H-2*^{b}), B10.129 (5M)Cr(*H-2*^{b}, *H-1*^{b}), C57Bl/10 ScSnCr (*H-2*^{b},*H-1*^{c}), BALB/c Cr (*H-2*^{d}), C_3H Cr (*H-2*^{k}), SJL (*H-2*^{s})

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strain; hybrid male mice BALB/cCr × DBA/2Cr F_1 ($H\text{-}2^d/H\text{-}2^d$, hereafter called $CD2F_1$) 2–3 months old; male mice maintained in a close but not inbred colony of the strain BALB/c/A/BOM Cr($H\text{-}2^d$, nu/nu, genetically thymusless, "nude" strain on BALB/c background, originally obtained from Dr. Carl W. Friis, Ry, Denmark, hereafter referred to as BALB-Nu); and 5-month old male rats of Sprague–Dawley strain were used. All animals were obtained from the Animal Production Section, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Tumors

Ascitic L1210, L1210/AraC (L1210 subline resistant to AraC), P388 and $6C_3HED$ lymphomas and sarcoma 180 (S-180), solid Lewis lung carcinoma (LLC) and B16 melanoma (B16M) were obtained at the National Cancer Institute. Radiation-induced lymphoma L5MF-22 of B10.129(5M) origin [10] was kindly supplied by Dr. G. Cudkowicz (Department of Pathology, State University of New York at Buffalo, Buffalo, N.Y.). ATPC+, a BALB/c mammary carcinoma [11], GPY, a Polyoma-induced sarcoma of hamster origin, and HeLa of human origin, have been maintained in tissue culture in our laboratory for several years.

Ascites L1210, L1210/AraC and P388 leukemias were passaged in $CD2F_1$ male mice, $6C_3HED$ in male C_3H recipients, and S-180 in male swiss mice, (10^5 cells i.p., suspended in 0.2 ml medium 199, weekly). LLC and B16M lines were maintained by injecting 10^6 viable cells (in medium 199) obtained from solid tumors, into the hind leg of female C57Bl/6 mice. Splenic lymphoma L5MF-22 was serially passaged using cell suspension obtained from grossly enlarged spleens, injected intravenously (10^5 cells/mouse i.v., in medium 199) into syngeneic B10.129(5M) or compatible C57Bl/10 male mice [10]. ATPC+, GPY and HeLa cells were serially passed in tissue culture (see below).

In vitro *incubation of normal or tumor cells for UIF production*

Normal spleen or splenic tumors were teased with a sterile flat-end forcep in order to obtain a cell suspension in chilled medium 199 (Difco Lab., Detroit, Michigan). Cell suspension was washed three times centrifuging at 250 × ***g*** for 10 min and resuspended in the same medium at the desired cell concentration. Since cell washing did not modify the results, in some experiments the entire washing procedure was omitted. Ascites tumors were collected from the peritoneal cavity of mice, diluted with medium 199, washed twice centrifuging at 250 × ***g***, and resuspended at the desired concentration in medium 199. Incubation of cell suspensions was carried out overnight for 16 hr at 37°C in a CO_2 incubator, 10% CO_2, in 50 mm dia sterile Petri dishes (Falcon Plastics, Oxnard, Ca.). After incubations the supernatant was collected, centrifuged at 400 × ***g***, dispensed into sterile vials and stored at −20°C until use. When supernatant of frozen-thawed cells were used, spleen cell suspension (20×10^6/ml) in medium 199 was frozen at −70°C and thawed at 39°C, three times and then centrifuged at 400 × ***g*** for 15 min and the supernatant stored at −20°C until use.

$^{125}IUdR$ uptake assay

Bone marrow cells obtained from femurs and tibias of donor mice, or splenic or ascites tumor cells were suspended in chilled medium 199, washed three times as previously described and resuspended at the concentration of 2.5×10^6 cells/ml of medium 199. Four tenths of a millilitre of cell suspension (10^6 cells) was incubated with 0.4 ml of medium 199 or of supernatant of overnight-incubated cells at various concentrations in plastic test tubes (12 × 75 mm, Falcon Plastics) for 1 hr at 37°C in a CO_2 incubator, in 10% CO_2. Then 0.2 ml of medium 199 containing 50% of heat-inactivated fetal calf serum (FCS, GIBCO, Grand Island, N.Y.), 0.1 μg of 5-fluoro-2′-deoxyuridine (FUdR) and 1 μCi of ^{125}IUdR, were added, and the tubes incubated further for 2 hr in the same conditions previously described. Cell suspension was then washed three times with chilled medium 199, centrifuging at 250 × ***g***. After the last centrifugation, the supernatant was discarded and the pellet read in a γ-scintillation counter (Packard, Gamma Scintillation Spectrometer, Model 3002).

When plasma of mice was tested, 0.4 ml of plasma from control or tumor-bearing mice was added to 0.4 ml of L1210 cell suspension and the test performed as previously described. Plasma from $CD2F_1$ mice challenged with 10^5 cells i.p. of L1210, L1210/AraC and P388 lines, from C_3H mice challenged with 10^5 cells i.p. of $6C_3HED$ line, and from B10.129(5M) inoculated with 10^5 cells i.v. of L5MF-22 lymphoma was collected 7 days after tumor transplantation. Plasma from C57Bl/6 mice inoculated with 10^5 cells i.m.

of Lewis lung carcinoma or B16 melanoma was collected 12 days after tumor challenge.

UIF determination

The inhibitory activity of supernatants of cells incubated overnight was assayed using L1210 leukemia cells, except when otherwise specified in the test.

Inhibition of ^{125}IUdR uptake by tumor cells mixed with splenocytes in vitro

Tumor cell suspension in 0.4 ml of medium 199 was added to plastic tubes (12 × 75 mm, Falcon Plastics) containing 0.4 ml of washed spleen cell suspension in medium 199 obtained from mice lethally irradiated (800R) 1 day before cell collection. After 1 hr and 45 min of incubation in a CO_2 incubator (37°C, 10% CO_2), 0.1 ml of heat-inactivated fetal calf serum (GIBCO) and 0.2 ml of medium 199 containing FUdR 0.1 μg and ^{125}IUdR 1 μCi were added. Following 16 hr of incubation as previously described, cell mixtures were washed three times and the pellet read in the γ-scintillation counter (Packard).

Tissue cultures of tumor cells

ATPC+, GPY and HeLa cells were cultivated either in plastic test tubes (12 × 75 mm, Falcon Plastics) or in Petri dishes (50 mm dia, Falcon Plastics) using Eagle medium (Difco Laboratories) containing 10% heat-inactivated calf serum and penicillin and streptomycin. The number of cultured cells was determined by detaching cells with a rubber policeman and counting them in a hemocytometer chamber in presence of trypan blue.

Irradiation procedures

Mice received a total-body irradiation of 800 rad using a Westinghouse "Quadrocondex" X-ray machine; 200 kV, 15 mA, at a distance of 54 cm, using 0.25 mm copper and 0.5 mm aluminum filters, at a dose rate of 137 rad/min. Cell suspensions in medium 199 were irradiated *in vitro* with 5000 rad in sealed glass vials at room temperature, using the same X-ray machine, at a distance of 25 cm, at a dose rate of 661 rad/min.

Reagents and drugs

Silica particles were obtained through the courtesy of Dr. G. Cudkowicz (Dept. of Pathology, School of Medicine, State University of New York at Buffalo, Buffalo, N.Y.). Actinomycin D and 5-fluoro-2′-deoxy-uridine were obtained from Drug Synthesis and Chemistry Branch, National Cancer Institute, NIH; ^{125}IUdR was obtained from Radiochemical Centre (Amersham, England); pepsin and papain were obtained from Sigma (St. Louis, Mo.) and pronase from Calbiochem (Lajolla, Calif.).

IDF [Immunodepressive factor(s)] preparation [12]

Cell suspension (10^8 cells/ml) in medium 199 of IDF-positive L5MF-22 lymphoma line, was frozen and thawed 3 times, centrifuged at 1000 × ***g*** for 20 min, the supernatant collected, diluted 1:10 with medium 199 and filtered through a 0.40 μ-millipore sterile membrane and stored at −20°C. Donor mice were treated with 0.2 ml/mouse i.p. of IDF preparation (final dilution = $1:10^4$) three days before the spleen collection.

RESULTS

Two lymphomas that originated in mice carrying the *H-2*^{b} (L5MF-22) or *H-2*^{d} (L1210) haplotypes respectively were cocultured *in vitro* for 16 hr with spleen cells collected from lethally-irradiated donors compatible or not for the *Hh-H-2* complex [1–8]. The data (Table 1) indicate that strong inhibition of ^{125}IUdR uptake occurred in tumor cells cocultivated either with compatible or allogeneic *Hh*-incompatible splenocytes. These data suggested the possibility that soluble, non-specific inhibitor(s) of ^{125}IUdR uptake could have been released by spleen cells, following short term culture *in vitro*. Therefore several viable or non-viable spleen cells were incubated either at 37°C or at 4°C, the supernatant collected and tested for inhibitory activity on ^{125}IUdR uptake by L1210 leukemic cells *in vitro*. The data illustrated in Fig. 1 show that the supernatant of viable mouse or rat spleen cells incubated at 37°C inhibited markedly the ^{125}IUdR uptake, whereas no inhibitory activity was found when heat-killed cells were incubated at 37°C or viable cells were incubated at 4°C. The supernatant of frozen–thawed cells incubated overnight at 37°C showed marginal activity, whereas no activity was found when medium 199 alone was used.

A dose–response assay was performed using serial dilutions of supernatant from incubated B10.129(5M) spleen cells (12×10^7 cells/ml), and L5MF-22 target cells. Inhibition of ^{125}IUdR uptake by tumor cells was proportional to the concentration of supernatant added, and an S-shaped curve was obtained. The data reported on probability chart gave a straight line, as evidenced in Fig. 2. Similar results, not reported here, were obtained when

Table 1. *Uptake of* ^{125}*IUdR by tumor cells mixed with normal spleen cells* in vitro

Tumor cells			Spleen cells	
Type	No. cells/ml	Donor* strain	No. cells/ml	Counts/min ± S.E.†
L5MF-22	10^5	—	—	7863 ± 1661
L5MF-22	10^5	C_{57}Bl/6	10^7	916 ± 98
L5MF-22	10^5	BALB/c	10^7	524 ± 38
L1210	10^5	—	—	3776 ± 1011
L1210	10^5	C_{57}Bl/6	10^7	849 ± 74
L1210	10^5	BALB/c	10^7	465 ± 28
—	—	C_{57}Bl/6	10^7	597 ± 57
—	—	BALB/c	10^7	547 ± 40

*Irradiated with 800 rad, 1 day before spleen collection.
†Counts/min of washed cell pellet ± standard error.

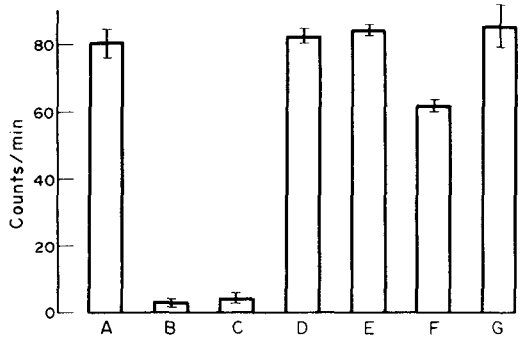

Fig. 1. ^{125}*IUdR uptake by L1210 cells* (10^6 cells/ml) *incubated* (3 hr *at* 37°C) *with supernatants* (1:1) *of short-term cultures* (16 hr *at* 37°C, *except for "E") of spleen cells* (10^7 cells/ml). *Counts per minute of L1210 cell pellet. Arithmetic mean of* 2 *samples* ± *standard error (bars).*
A—Control, L1210 cells alone. B—L1210 cells incubated with supernatant of CD2F$_1$ *spleen cell culture. C—L1210 cells incubated with supernatant of spleen cells of Sprague–Dawley Rats. D—L1210 cells incubated with supernatant of cell culture of heat-killed* (1 min *at* 90°C) *CD2F*$_1$ *spleen cells. E—L1210 cells incubated with supernatant of CD2F*$_1$ *spleen cell culture kept* 16 hr *at* 4°C. *F—L1210 cells incubated with supernatant of cell culture of frozen–thawed CD2F*$_1$ *spleen cells. G—L1210 cells incubated with medium* 199 *kept* 16 hr *at* 37°C.

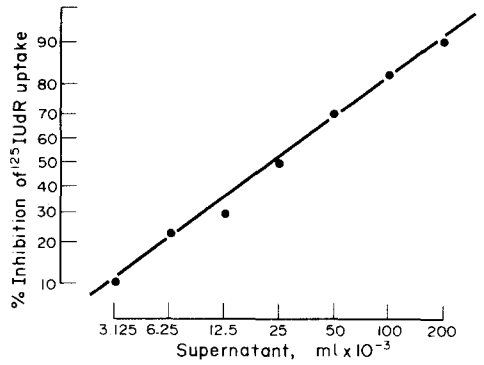

Fig. 2. *Inhibitory activity of supernatant of B*10.129 (5M) *spleen cell culture* (12×10^7 cells/ml, 16 hr *at* 37°C) *on* ^{125}*IUdR uptake by L5MF-22* (10^6 cells/ml) *lymphoma cells. Each point represents the percentage calculated on the arithmetic mean of two samples.*

L1210 leukemic target cells were used for the ^{125}IUdR uptake inhibition assay. On the basis of such data, it was possible to define the UIF unit as the amount of inhibitor(s) present in the supernatant, capable of inhibiting the ^{125}IUdR uptake of 10^6 normal or tumor cells by 50%. Under this definition, the preparation used in the assay reported in Fig. 2 contains approximately 40 UIF units/ml, against L5MF-22 lymphoma cells.

UIF production by normal or neoplastic cells of the same genetic origin, was determined using splenocytes pooled from ten B10.129(5M) male mice or L5MF-22 lymphoma cells (of B10.129(5M) origin) incubated for 16 hr at various cell concentrations as illustrated in Fig. 3. UIF was assayed against L1210 leukemia

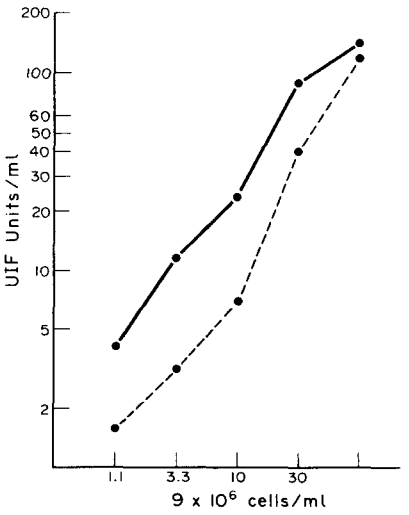

Fig. 3. *UIF production by normal or lymphoma cells of the same genetic origin. incubated* 16 hr *at* 37°C *at various concentrations.*
*Solid line: normal spleen cells of B*10.129(5M) *female mice.*
*Dotted line: L5MF-22 lymphoma cells (of B*10.129(5M) *origin).*
Each point represents the arithmetic mean of two samples.

cells and the results indicate that UIF production was proportional to cell concentration. The activity of normal spleen cells was slightly higher than that of tumor cells.

The production of UIF by splenocytes of various strains of mice and by a number of mouse tumor lines was determined. The results reported in Table 2 show that normal spleen cells of various conventional strains of mice or of athymic mice produced comparable amounts of UIF. On the other hand marked variation of UIF production was found in tumor lines, ranging from 2.83 to 46.39 units/ml. It is noteworthy that UIF was produced by splenic and ascitic tumors as well, and that a minimal amount of UIF was produced by parental L1210 line, whereas high levels of the inhibitor were produced by the Ara C-resistant leukemia subline.

The inhibitory activity of two UIF preparations on ^{125}IUdR uptake by normal bone marrow or various tumor lines was tested and the results are reported in Table 3. In general lymphoma and normal bone marrow cells were more sensitive to UIF than carcinoma, melanoma or sarcoma tumors. The only exception was the GPY line, a hamster sarcoma of Polyoma virus origin, maintained in tissue culture.

Preliminary studies were conducted in order to determine whether UIF would have any inhibitory effect on cell replication *in vitro*.

Table 2. Release of UIF by normal or tumor cells cultivated in vitro *for* 16 hr *at* 37°C (10^7 cells/ml)

Normal spleen cells			Tumor cells		
Donor strain	*H*-2 haplotype	UIF units/ml	Tumor	*H*-2 haplotype	UIF units/ml*
C_{57}B1/6	b	22	L5MF-22	b	5.6±1.9(2)†
B10.129(5M)	b	17.7±3.2(4)	L1210	d	2.8±0.9(3)
BALB/c	d	12.6	L1210 Sp‡	d	9.8
BALB/Nu	d	20.4±5.7(3)	L1210/Ara C	d	31.4
$CD2F_1$	d/d	13.6±5.4(5)	L1210/DTIC	d	9.9
SJL	s	17.3	LSTRA	d	24.1
			LPC-1	d	46.4

*1 unit = amount of UIF capable of inducing 50% inhibition of ^{125}IUdR uptake by 10^6 L1210 leukemic cells.

†Mean ± standard error of more than 1 UIF preparation (in parenthesis the number of UIF batches tested).

‡Splenic L1210 leukemia obtained from mice injected with 10^5 L1210 cells i.v. Leukemic spleen cells were used.

Table 3. Inhibitory activity of UIF on ^{125}IUdR uptake by normal bone marrow or various tumor cells

UIF batch No.§	Test cells	UIF units/ml‖	UIF % activity¶
1	L1210 leukemia	11.6	100.0
1	Bone marrow ($CD2F_1$)	6.96	60.0
1	Lewis lung carcinoma	2.5	21.6
1	B16 melanoma	1.6	13.8
1	Sarcoma 180	1.2	10.3
2	L1210 leukemia	160.3	100.0
2	L5MF-22	65.7	40.9
2	ATPC+	22.2	13.8
2	GPY	330.0	205.9
2	HeLa	22.2	13.8

§ Batch No. 1 was obtained from $CD2F_1$ cultured spleen cells (10^7 cells/ml), and Batch No. 2 from C_{57}B1/6 cultured spleen cells (14×10^7 cells/ml).

‖ 1 unit = amount of supernatant capable of inhibiting 50% of ^{125}IUdR uptake of test cells.

¶ % activity related to the inhibitory activity on ^{125}IUdR uptake by L1210 cells (= 100%).

The total number of ATPC+, GPY and HeLa cells was significantly lower in the cultures exposed to UIF in various experimental conditions as illustrated in Table 4. In particular UIF was more effective when added to the culture on the same day of cell seeding than 1 or 2 days later (group 1 vs 2, group 6 vs 7), with one exception (group 4 vs 5) where essentially total inhibition of tumor growth was found in both cultures.

Experiments were performed in order to test whether physical or chemical agents would have any influence *in vivo* or *in vitro* on UIF production by normal spleen cells. The results, illustrated in Table 5, show that immunosuppressive doses of X-rays, lethal for

Table 4. *Effect of UIF on cell proliferation*

Group No.	Cell type*	UIF† Units/ml	UIF† Day‡	Cell inhibition Day of test§	Cell inhibition Decrease‖ (%)	Cell inhibition Test¶
1	ATPC+	1.0	0	2	65.6	T
2	ATPC+	1.0	1	2	48.4	T
3	ATPC+	7.3	0	3	52.8	P
4	ATPC+	10.0	0	2	99.1	T
5	ATPC+	10.0	1	2	100.0	T
6	ATPC+	19.0	0	3	100.0	P
7	ATPC+	19.0	2	3	65.4	P
8	GPY	3.3	0	1	37.8	P
9	GPY	6.6	0	1	52.7	P
10	HeLa	9.8	1	2	47.2	P

*20,000 or 30,000 cells/plate or per tube.
†Obtained from C_{57}Bl/6 spleen cells.
‡Time when UIF was added to the culture at the final concentration reported in units/ml.
§Time when cells in culture were counted.
‖Decrease in the number of cells, respect to the controls incubated without UIF.
¶Test carried out in test tubes (T) or in plates (P).

Table 5. *Influence exerted by various agents on the production of UIF by mouse spleen cells* (10^7 cells/ml, 16 hr *at* 37°C)

Treatment	*In vivo* Host	*In vivo* Time**	*In vitro* Donor strain††	UIF activity units/ml
X-rays (800 rad)	C_{57}Bl/6	day-1	—	22
X-rays (800 rad)	BALB/c	day-1	—	80
Silica (5 mg/mouse)	BALB/c	−3 hr	—	19.9±0.5 (17.8±4)‡‡
IDF§§	BALB/c	day-3	—	11.7 (11.8)‖‖
X-rays (500 rad)	—		CD_2F_1	3.2 (5.2)¶¶
X-rays (5000 rad)	—		B10.129(5M)	11.6 (11)¶¶
X-rays (5000 rad)	—		SJL	14.9 (12.2)¶¶
Act D (1 μg/ml)	—		$CD2F_1$	33.7 (33.7)¶¶
Act D (0.1 μg/ml)	—		$CD2F_1$	24.4 (33.7)¶¶
FUdR (0.1 μg/ml)	—		$CD2F_1$	20 (40)
FUdR (1 μg/ml)	—		$CD2F_1$	17.6 (40)
Act D (1 μg/ml)	—		No spleen cells	5.4***
Act D (0.1 μg/ml)	—		No spleen cells	1.3***
FUdR (1 μg/ml)	—		No spleen cells	1.26***

** Time related to that of spleen removal.
†† Strain of the mice, from which spleen cells were collected.
‡‡ Mean of 4 pools of 2 spleens each ± standard error (in parenthesis the mean of 4 pools of 2 spleens each of control BALB/c mice not pretreated with silica).
§§IDF, immunodepressive factor [10], 0.2 ml/mouse i.p. diluted $1:10^4$).
‖ ‖ In parenthesis UIF activity of untreated BALB/c control spleen cells.
¶¶ In parenthesis UIF activity of the untreated control portion of spleen cell suspension.
*** Act D or FUdR in medium 199 incubated overnight at 37°C.

Table 6. Effect of various treatments in vitro *on UIF activity*

Treatment*	UIF (units/ml) Untreated	Treated	Change of UIF activity (%)
Heat: 56°C (30 min)	47	31.1	−33.8
100°C (30 min)	47	23.3	−50.4
Pepsin 500 μg/ml	65.1	48	−26.3
Papain 500 μg/ml	65.1	50.3	−22.7
Pronase 500 μg/ml	77.7	54.5	−29.9
Pronase 50 μg/ml	77.7	58.6	−24.6
Pronase 5 μg/ml	77.7	74.3	−4.4

*The enzyme treatment was carried out for 30 min at 37°C. Values of inhibitory activity on ^{125}IUdR uptake due to enzymes alone were subtracted in order to calculate UIF activity of enzyme-treated samples.

the donor (800 rad), or lethal for lymphoid cells *in vitro* (5000 rad) did not prevent UIF production. An antimacrophage agent such as silica particles [13] and IDF, a virus capable of depressing T-dependent allograft reactions [10, 12], were not capable of impairing UIF production. On the other hand, treatment with FUdR, capable of inhibiting endogenous thymidine synthesis [14], decreased the UIF production by approximately 50%. Moreover a slight inhibitory effect seemed to have been induced by *in vitro* treatment with actinomycin D.

The sensitivity of UIF to heating or to treatment with proteolytic enzymes *in vitro* was studied, using a UIF preparation obtained from $CD2F_1$ spleen cells and tested against L1210 leukemic cells for ^{125}IUdR uptake inhibition (Table 6). The data show that UIF was relatively resistant to heating: almost 50% of UIF activity remained after 30 min exposure to 100°C. A limited reduction (23–30%) of UIF titer was induced by enzymatic treatment *in vitro*, with all three proteolytic enzymes tested.

Since considerable amounts of UIF were produced by tumor cells *in vitro*, preliminary studies were performed in order to test whether plasma of tumor-bearing mice would contain appreciable amounts of UIF. Plasma of control or tumor-bearing mice was added to L1210 leukemic cells and incubated with ^{125}IUdR. UIF activity was calculated taking into account the ^{125}IUdR uptake of L1210 cells incubated with normal mouse plasma (100% of precursor uptake). The results of the experiment (Table 7) showed that appreciable levels of UIF activity were detectable in tumor-bearing mice and ascitic fluid.

Table 7. Inhibitory activity of plasma of tumor-bearing mice on ^{125}IUdR uptake by L1210 leukemia cells (10^6/ml) in vitro

Host strain	Tumor	UIF (units/ml†
$CD2F_1$	L1210	7.7
$CD2F_1$	L1210	36‡
$CD2F_1$	L1210/AraC	14
$CD2F_1$	P 388	8.9
C_3H	6 C_3HED	11.7
C_{57}Bl/6	Lewis lung carcinoma	7.3
C_{57}Bl/6	B16 Melanoma	6.3
B10.129(5M)	L5MF-22	18

† In plasma of tumor-bearing mice.
‡ Units/ml of ascitic fluid.

DISCUSSION

In the course of studies on anti-*Hh* reactions *in vitro*, using lethally-irradiated spleen cells, soluble, non-specific inhibitor(s) (UIF) of ^{125}IUdR uptake by normal and cancer cells has been detected. A number of biological properties of UIF has been studied and they can be outlined as follows:

(a) UIF is produced by non-stimulated normal spleen as well as cancer cells;
(b) UIF is non-strain, non-species or tissue specific, since it was produced by tumors and spleens of various strains of mice (Table 2) as well as by rat spleen cells (Fig. 1) and was found to be active on normal or tumor cells of various origin (Table 3);
(c) UIF production is not conditioned by mature T cells activity, being synthesized actively by spleen cells of nude mice (Table 2);

(d) at high concentration UIF seems to be cytotoxic or cytostatic for various tumor lines in tissue culture (Table 4);
(e) lethal irradiation both *in vivo* and *in vitro*, silica or IDF treatment *in vivo*, capable of interfering with macrophage functions or with antilymphoma allograft reactions respectively [12, 13] did not prevent UIF production (Table 5);
(f) inhibition of endogenous thymidine synthesis by FUdR reduced but did not abolish UIF production (Table 5);
(g) protein-synthesis inhibition, induced by treatment with actinomycin D *in vitro*, did not prevent UIF production (Table 5);
(h) UIF is relatively stable to heat and proteolitic enzyme treatment *in vitro* (Table 6);
(i) UIF-like activity can be detected in the blood of tumor-bearing mice (Table 7).

A number of factors released by human [15–18], mouse [19–25], hamster [26], rat [27, 28] and calf [27, 29] normal or stimulated lymphoid cells have been described in the literature. Some of them were found to be active in inhibiting thymidine incorporation or proliferation of cancer cells [15, 19, 21, 23, 24] as well as of normal lymphocytes [15, 17, 19, 21–29]. Immunosuppression and cytotoxic activity was demonstrated for extracts of calf thymus and spleen [29] and for a factor obtained from Con A-stimulated splenocytes of $C_{57}Bl/6$ mice (SIRS, 22). Nonstimulated, or Con A, LPS, PHA-stimulated cultures of splenocytes of various strains of mice produce a proliferation inhibiting factor (PIF) which is T-dependent and species-specific [23]. Hersh *et al.* [18] suggested that cell "crowding" in culture would result in release of soluble "signals" of proteinaceous nature capable of inhibiting DNA synthesis. More recently Opitz *et al.* [30], Fernbach *et al.* [25] and Evans and Booth [31] pointed out that a number of inhibitors described in the literature should not be considered to be true inhibitors of DNA synthesis, but as small molecules capable of interfering with the uptake of the labeled thymidine or ^{125}IUdR used for the assay. A good candidate was considered to be thymidine itself [30], capable of competing with the labeled compound for incorporation into DNA. On the other hand Fernbach *et al.* advanced the hypothesis of the contemporary presence of two factors, i.e. thymidine itself and a true inhibitor of DNA synthesis, although they suggested also that excess of thymidine could produce DNA-synthesis inhibition [25].

If the properties of UIF are taken into account, it seems difficult to assess possible identity or non-identity of UIF with any of the factors described in the literature. In fact few data are available concerning similar factors produced either by normal lymphoid and cancer cells *in vitro*. Of particular interest for its analogy with UIF seems to be the dializable factor capable of inhibiting bone marrow colony formation described by Metcalf [19]. The material was not species specific, was heat-resistant and was produced by cultured normal bone marrow, thymic spleen or lymph node cells as well as by leukemic cells. On the other hand UIF production was depressed by FUdR (Table 5), an inhibitor of the biosynthesis of endogenous thymidine [14]. This observation seems to confirm the hypothesis advanced by Opitz *et al.* [30], that UIF activity would be, at least in part, due to thymidine released by lymphoid or tumor cells in tissue culture. Nevertheless UIF was partially heat-labile and reduced in its activity by proteolytic enzymes In particular a plateau was found when graded doses of pronase were used (Table 6), being equally active at 50 μg/ml and at 500 μg/ml in reducing UIF titers. This opens the possibility that UIF might be a complex of soluble factors, including a heat stable factor not destroyed by proteolytic enzymes, which could well be thymidine, and other heat-labile enzyme-sensitive factor(s) of possible protein nature, capable of interfering with proliferation of normal or tumor cells.

The biological importance of UIF is difficult to establish. No relationship was found between UIF production and strain reactivity against *Hh* alloantigens. In fact spleen cells of anti-*Hh* high responder [B10.129(5M) [3, 4] and BALB-Nu [6, 8, 32]] or low responder [SJL [4]] strains produced comparable amounts of UIF (Table 2). However, no studies were conducted with the heat-labile or heat stable fraction of UIF, in order to test whether such relationship could be found with one of these two fractions.

In addition impairment of T-dependent or T-independent immune responses by *in vivo* or *in vitro* lethal irradiation [8], i.v. administration of silica particles [13], or pretreatment of donor mice with IDF [10, 12] did not prevent UIF production by splenocytes.

Nevertheless we cannot exclude that UIF could play a role in cell proliferation inhibition in antitumor immune responses [5, 8, 33, 34] and give account, at least in part, for the decline of tumor cell proliferation in

lymphoma-bearing mice close to leukemic death [9]. In any case when *in vitro* tests involving cell proliferation monitored by the uptake of DNA-labeled precursors are carried out, the possible production of UIF should always be taken into consideration. The presence of UIF in the assay medium could result in significant interference with uptake of precursors, leading to misinterpretation of the results.

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The Effect of Neonatal Thymectomy on the Growth Kinetics of a Virus-induced Renal Sarcoma of the Rat*

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Abstract—*Growth kinetics of Polyoma virus induced renal sarcomas in normal and neonatally thymectomized Wistar rats are compared. Both groups of animals show an exponential tumour growth from the* 20th *to the* 50th *day of life. Thymectomized rats have a significant reduction of tumour doubling time* (76 hr) *compared to non-thymectomized rats* (110 hr). *The main parameters of tumour growth, cell cycle time, proliferative fraction and cell loss rate are determined at the* 30th *and* 50th *day after virus infection of the animals. The reduction of doubling time in tumours of thymectomized rats is due to an increase of the proliferative fraction despite increased cell loss rates. The extent of cell loss in the resting and cycling cell compartment is specified for both groups of rats.*

INTRODUCTION

THE INFLUENCE of early thymectomy upon the incidence of virus induced tumours, especially by Polyoma virus has been known a long time [1–9]. Furthermore it was shown that after infection of Wistar rats with Polyoma virus the number of distinct tumour foci at the beginning of cellular proliferation and also the tumour mass as a whole in one kidney was significantly increased in thymectomized animals [10]. The growth kinetics of these fibrosarcomas in normal immune-competent rats had been studied previously [11]. Therefore it was interesting to compare their kinetics to the growth of sarcomas in immune-deficient thymectomized rats by a complete analysis of all important kinetic parameters. This comparison should bring some more understanding for the striking difference in growth kinetics of Polyoma virus induced tumours between thymectomized and normal rats.

MATERIAL AND METHODS

1. *SE-Polyoma virus*

Cultivation and preparation of the virus has been described previously [11, 12]. The $TCID_{50}$ of the virus suspension used was 10^6 or 10^7 per 0.3 ml. HA-titre: 768.

2. *Animals*

Non-inbred male and female Wistar rats from the conventional colony strain of the Breeding Institut for Experimental Animals, Hannover, West Germany.

One group of animals was inoculated with 0.3 ml virus suspension s.c. within 24 hr. after birth. A second group was thymectomized before virus inoculation using the same dose and virus preparation. Thymectomy was performed within the first 24 hr. post partum by the method of Martinez [13]. If any thymus gland was found macroscopically or histologically after sacrifice the animal was excluded from further evaluations.

3. *Histomorphometrical determination of tumour weights*

One hundred and forty-nine non-thymectomized animals were sacrificed between the 25th and 62nd day of life, 1–6 animals (3 on average) per day. The blood volume was reduced by cardiac puncture before nephrectomy.

The fresh kidneys were cleaned from adhering tissue and weighed on an analytic scale. Further procedure see Zobl *et al.* [11].

The total tumour weight was determined by

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the equation:

$$W_t = W_k \times A_t / A_k; \quad \text{(I)}$$

W_t: weight of tumour;
W_k: weight of kidney;
A_t: area of tumour;*
A_k: area of kidney.*

The specific weight of the kidneys with or without tumour is between 1.0 and 1.1.

Seventy-seven thymectomized animals inoculated with the same virus preparation as the non-thymectomized ones were sacrificed between the 25th and 60th day of life, 1–4 animals (2 on average) per day. The tumour weights were determined analoguously.

4. *Historadioautography with* 3H-*thymidine*

The autoradiographic technique has been described previously as well as the evaluation of the autoradiograms [11]. The following kinetic parameters were determined by this technique in 30- and 50-day old animals:

(a) Labelling index 1 hr after a pulse of 3H-thymidine (LI1h). Dosage: 1 μCi/g body weight; specific activity: 5 Ci/mmole.

(b) Cell cycle times and phase durations including time distributions by the %-labelled mitoses method [14]. The evaluation of the PLM-data was made by computer analysis [Dr. G. G. Steel-15].

(c) Proliferative fraction (PF) from PLM-data following method of Lala and Patt [16].

(d) Cell production rate (CPR), cell loss rate (CLR) and mode of cell loss specifying loss from proliferative (PCL) and non-proliferative compartment (QCL) determined by continuous labelling with 3H-thymidine. Details of infusion technique see Eve and Robinson [17]. Dosage: 1 μCi/g body weight per 24 hr; Specific activity: 5 Ci/mmole. Continuous infusion was done in 190 non-thymectomized and 90 thymectomized rats. The labelling index was determined every 2 hr between 46 and 76 hr after the start of labelling. On average 2 tumours were evaluated at each point of time as far as sufficient labelling was found in the autoradiograms. Three to four thousand cells were counted per tumour within a representative area independently by 2 observers.

RESULTS

Twenty days after virus inoculation in new born rats sarcomatuous neoplasms are histologically recognizable in the kidneys with great consistancy in both thymectomized and non-thymectomized animals [8]. In this study tumour kinetics of 30 and 50-day old animals, i.e. 10- and 30-day old sarcomas are described. In the following, the age of tumour bearing animals will be given exclusively.

(1) *Tumour incidence*

From 149 non-thymectomized rats 41 unilateral and 65 bilateral renal sarcomas were obtained, a yield of 171 tumours in 298 kidneys equal to 57.5%. In 77 thymectomized rats 17 unilateral and 44 bilateral sarcomas were found, a yield of 105 tumours in 154 kidneys equal to 68.5%.

(2) *Growth curve*

In Fig. 1 the logarithms of tumour weights ($\log_{10}$, mean values) are plotted against the age of animals between the 25th and 60th day of life. Up to the 50th day there is an increase of tumour weights for both groups of animals. Thereafter a slow-down of growth or even an incipient regression is observed. Because of the wide spread of the tumour weights a simple exponen-

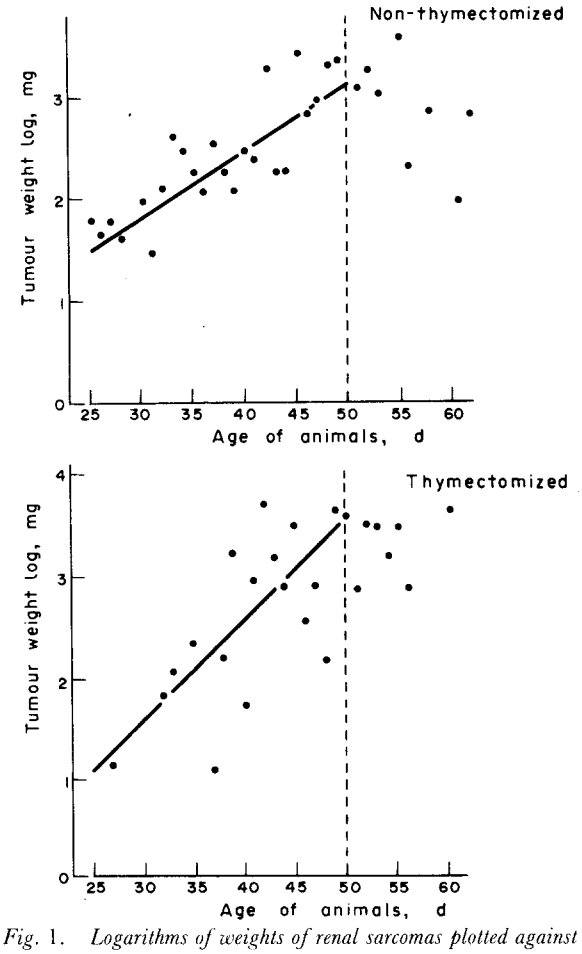

Fig. 1. Logarithms of weights of renal sarcomas plotted against the age of non-thymectomized and thymectomized tumour bearing rats. Points represent mean values of 2–6 tumours. Regression analysis performed between the 25th and 50th day. The regression lines are entered into the Fig. indicating a tumour doubling time of 110 hr *in non-thymectomized and* 76 hr *in thymectomized animals.*

* Determined at a level of greatest diameter of tumour in serial sections through the kidney.

Table 1. Labelling index 1 hr after a pulse of 3H-thymidine (LI1h) into rats with PV-induced renal sarcomas. Proliferative fraction (PF) calculated by method of Lala and Patt [16]. Potential tumour doubling time (T_{dpot}) calculated following Steel [18]. Actual tumour doubling time (T_d) estimated by a histomorphometrical experiment

Age of animals (days)	LI(1h) %	PF %	T_{dpot} (h)	T_D
30	13	42	105	110
30*	13	55	43	76
50	15	48	55	110
50*	18	58	38	76

* Tumours of thymectomized animals.

tial function was fitted to the experimental data from the 25th to the 50th day. The regression coefficient of the tumour weights of non-thymectomized animals is $W_1 = 0.0655$ (± 0.007) and of thymectomized animals $W_2 = 0.0951$ (± 0.002). The respective regression lines are entered into Fig. 1. Therefore tumour doubling time (T_d) is calculated amounting to 110 hr in the non-thymectomized group and 76 hr in the thymectomized group (see Table 1). A significant difference is calculated in the *t*-test ($P = 0.05$).

(3) *Cell cycle times and phase durations*

The results of the PLM-experiments are shown in Table 2 and Fig. 2. A computer analysis was performed (by Dr. G. G. Steel), distributions of cycle times being part of evaluation (Fig. 3). Because of the high standard deviations significances were not tested. The older tumours of both groups show a wider spread of their cycle times especially towards the long end of individual cycle times.

(4) *Proliferative fraction*

PF was calculated by the equation:

$$PF = LI_{experimental}/LI_{theoretical} \qquad (II)$$

following Lala and Patt [16]. The values of LI (1 hr), PF and potential tumour doubling time (T_{dpot}) derived by method of Steel [18] are given in Table 1.

(5) *Cell production rate and cell loss*

The cell production rate (CPR) was calculated by the known values of PF, $\bar{T}_C$ (mean cycle time) and data obtained by a continuous labelling experiment. Additionally, continuous labelling data and the known actual tumour doubling time (T_d) give the cell loss rate (CLR) and the mode of cell loss. The latter specifies to what extent proliferative and resting cells are affected by loss.

The evaluation of continuous labelling data was done by method of Lang *et al.* [19]. According to it, the theoretical increase of LI in a growing cell population under continuous labelling is given by the equation:

$$LI_t = 1 - (1 - a)\exp[\phi(-T/\bar{T}_C)]; \quad (III)$$

LI_t : expected labelling index at a time t;
a : proliferative fraction (PF);
T : duration of 3H-thymidine labelling;
$\bar{T}_C$: mean cell cycle time.
The value e^{ϕ} was determined by an optimal fit of the model function (III) to the experimental data. ϕ includes the following parameters (calculation see Lang *et al.* [19]):
β : cell production rate (CPR);
δ_P : loss rate of P-compartment (PCL):
δ_Q : loss rate of Q-compartment (QCL);
In Table 3 cell production and loss rates are

Table 2. Cycle times, phase durations estimated by the labelled mitoses technique in PV-induced renal sarcomas of 30- and 50-day old thymectomized and non-thymectomized rats (computer analysis by Dr. G. G. Steel)

Age of animals (days)	Phase durations and cycle times Mean values and standard deviation (h)			
	T_{G_1}	T_S	T_{G_2}	T_C
30	10.8 ± 12.5	7.8 ± 2.6	2.5 ± 1.1	21.1 ± 12.8
30*	14.8 ± 10.8	7.0 ± 1.7	2.9 ± 1.3	24.7 ± 10.5
50	16.3 ± 14.3	10.5 ± 5.1	3.0 ± 2.5	29.8 ± 15.4
50*	15.3 ± 12.4	10.5 ± 6.2	2.7 ± 1.2	28.5 ± 13.9

* Tumours of thymectomized animals.

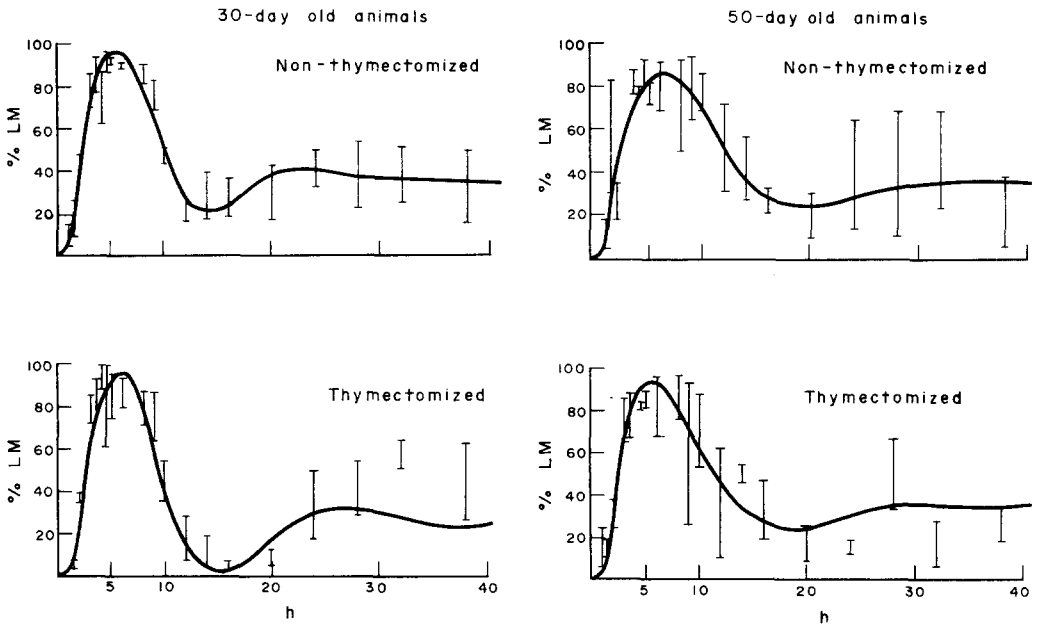

Fig 2. Percent-labelled-mitoses curves after pulse labelling with ^{3}H*-thymidine in tumours of* 30*-and* 50*-day old non-thymectomized and thymectomized rats. The curves are drawn following a computer analysis of the PLM-data (by Dr. G. G. Steel, London).*

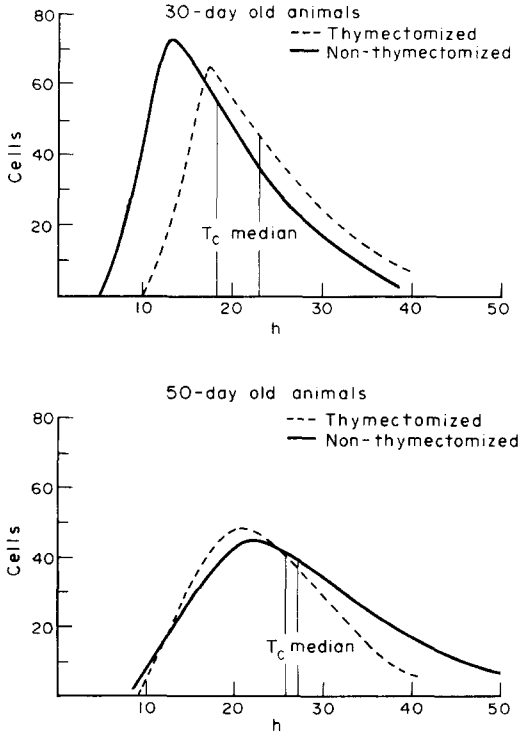

Fig. 3. Distribution of cell cycle times on the basis of computer analysed PLM-data in tumours of 30*- and* 50*-day old non-thymectomized and thymectomized rats. Vertical line: mean cycle time.*

given. Additionally, cell loss is given as a cell loss factor (CLF), i.e. the quotient of CLR and CPR in percentage. Finally, the P- and Q-cell loss per hour as a percentage of the respective compartment is differentiated and the relation between both rates added.

In younger animals the QCL is fairly higher than PCL especially in non-thymectomized animals. For older tumours no essential difference between QCL and PCL can be seen.

The PF and CPR are higher in sarcomas of thymectomized rats. An estimation of their standard deviations and a test of significance is not included. Since PF is determined by the use of PLM-data the phase durations with their wide standard deviations enter into the calculation and obscure a relatively low difference of PF values of about 10–15%. However, an increase of PF in thymectomized rats is to be accepted since only this parameter may explain the significant increase of the overall growth rate whereas cycle times are nearly identical and cell loss rates even higher in thymectomized animals.

For sarcomas of 50-day old thymectomized animals there is no satisfying fit of the model function (III) to the experimental data (see Fig. 4). That means PF obtained by the PLM-data is underestimated. A modification of loss parameters would not give a better fit. The remainder of the theoretical labelling curves shown in Fig. 4 give a satisfying accordance with the experimental values, i.e. reliable values of loss rates.

DISCUSSION

Thymectomy in new-born Wistar rats preced-

Table 3. Cell production rate and cell loss in PV-induced renal sarcomas of 30- and 50-day old thymectomized and non-thymectomized rats. The values are calculated by evaluation of continuous labelling data following method of Lang et al. [19], T_d *and PF. CPR: cell production rate per hour; CLR: cell loss rate per hour; CLF: cell loss fraction* $= CLR/CPR \times 100$; *PCL: P-cell loss as a percentage of total P-compartment lost per hour; QCL: Q-cell loss as a percentage of total Q-compartment lost per hour. QCL/PCL: quotient of absolute loss rates*

Age of animals (days)	CPR (1/h)	CLR (1/h)	CLF (%)	PCL (%/h)	QCL (%/h)	QCL/PCL
30	0.0066	0.0003	5	0.03	1.70	70
30*	0.0160	0.0069	43	0.63	1.02	1.7
50	0.0125	0.0062	50	0.56	7.12	1.3
50*	0.0184	0.0093	51	0.82	6.18	0.7

* Tumours of thymectomized animals.

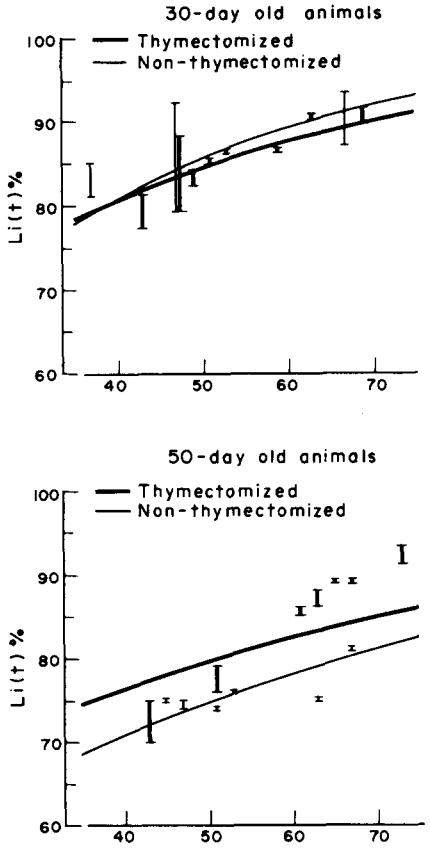

Fig. 4. Continuous labelling by intraveneous infusion of 3H-thymidine into 30- and 50-day old non-thymectomized and thymectomized rats. Experimental data with spread of values entered as vertical bars. The curves represent an optimal fit of a model function [19] *to the experimental data. The function renders the expected increase of the labelling index under continuous labelling.*

ing inoculation of Polyoma virus results in a significantly higher yield of renal sarcomas. This was already found in previous studies [8] and now confirmed within these experiments as an additional result. This is in accordance with the well known increase of tumour incidence in immune-deficient animals after viral or chemical tumour induction [1–9, 20–25].

The main conclusions from these studies may be characterized as follows: thymectomy increases the growth rate of sarcomas significantly whereas no remarkable influence on the timing of the cell cycle is seen. The accelerated growth rate is due to an increased PF. There is also no decrease of PF with increasing tumour age like in sarcomas of non-thymectomized hosts as previously shown [11].

The cell loss rate is essentially lower in tumours of non-thymectomized animals. This is remarkable because the retarded tumour growth of immune-competent rats may not be explained by an increased cell loss resulting from cytotoxicity of thymocytes as might be expected. It seems therefore that cell loss is caused merely by hypoxemia like in some further solid tumours [26–33]. The apparent lack of lymphocytes among the sarcoma cells in non-thymectomized rats may confirm this view.

There is another explanation for the different growth behaviour of tumours in thymectomized rats that could result from the depletion of T-lymphocytes. The immuno-deficiency might prolong the period of virus replication thereby extending the period of cell transformation. A sustained development of "new" tumour cells may provide a higher PF in contrast to immune-competent hosts. Still another factor affecting PF by immunological alterations is conceivable. The known influence of a stimulated immune system on G_2-cells [34] might be connected with a change of PF. Usually, this influence leads to a prolongation of the G_2-cycle phase or to preferential cell loss during this phase [35]. However, if a G_2-cell arrest would be suggested this could explain a reduction of PF too.

Changes of tumour cell kinetics under an altered immune situation of hosts are reported in

the pertinent literature [34, 36–39]. According to it, a retardation of the overall growth rate by immune pressure is known. These observations are comparable with our results indicating a decreased growth rate in immune-competent rats. Further parallels may be drawn with some solid tumours showing a decreased cell production rate after immunisation of the host [37] and a fairly good consistency of cell cycle times despite an immunological disturbance [34, 37]. Consequently, the change of cell production rate also results from a change of PF like in the renal sarcomas.

Interesting is a report on a solid tumour of rats treated with antithymocyte serum [38]. An increase of PF and decrease of T_d is observed in contrast to immune-competent hosts. So far it agrees well with the behaviour of the renal sarcomas. However, a substantially shorter cell cycle time in untreated rats is found leading to a reduced T_{dpot} and consequently to a higher cell loss factor.

Information about the mode of cell loss in tumours under immunological disturbance are sparse. Janik [35] reports a random loss from P- and Q-compartment under immune pressure. This finding is in accordance with our results except for small tumours of non-thymectomized rats with a predominant loss from the resting cell compartment.

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Interaction of Androgens with Oestradiol-17β Receptor Proteins in DMBA-Induced Mammary Tumours—a Possible Oncolytic Mechanism

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Abstract—*A series of androgens have been examined for their ability to* (1) *compete with* [^{3}H]*oestradiol-17β for the cytoplasmic oestradiol-17β receptor protein isolated from DMBA-induced mammary tumours and* (2) *facilitate the nuclear translocation of the oestrogen receptor. The most effective androgen examined was androst-5-ene-3β,17β-diol. Compounds that did not compete with* [^{3}H]*oestradiol-17β for cytoplasmic oestradiol-17β binding sites were unable to instigate the translocation of the oestrogen receptor. Androgen-transferred oestrogen receptors were found to have a relatively short nuclear retention time and readily bound* [^{3}H]*oestradiol-17β at* 4°C.

INTRODUCTION

A NUMBER of reports have demonstrated an oncolytic action for androgens in both human [1, 2] and rat [3–6] mammary tumours. The mechanism by which such compounds inhibit tumour growth has for the most part remained unknown. Since we have previously demonstrated that the growth of the DMBA-induced rat mammary tumour requires the presence of cytoplasmic oestradiol-17β receptor proteins [7–9] the tumour represents a good model system by which the hypothesis that the oncolytic effects of androgens are mediated via the oestrogen receptor system can be tested. The present study therefore examines the direct action of testosterone and other C_{19}-steroids on the binding of oestradiol-17β to its receptor protein in both cytoplasmic and nuclear compartments of DMBA-induced mammary tumours.

A preliminary report of this work has been presented [10].

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50±2 days old) by intubation with a single dose of DMBA (20 mg in 1 ml sesame oil). Animals were housed in groups of 4 and fed diet and water *ad libitum*. Beginning 5 weeks after intubation with DMBA, rats were palpated for tumours at weekly intervals and tumour size recorded as the mean of two perpendicular diameters, one measured across the greatest width. Tumours which did not attain an approximate size of 20 mm mean diameter in the 23 week period following carcinogen administration were not used in this study.

Preparation of subcellular fractions

Soluble supernatant (cytosol) preparations were obtained as described by Nicholson, Golder, Davies and Griffiths [8] using a homogenisation procedure resulting in maximum breakdown of tumour cell membranes with minimum damage to cell organelles [11]. Nuclei were prepared by a method described by Davies and Griffiths [12].

Competitive binding studies on cytoplasmic fractions

Aliquots (200 μl) of tumour cytosol preparations (approximately 20 mg protein/ml) from ovariectomised animals (operation performed one day previously) were incubated for 2 hr at 4°C with equal vol of 50 mM-Tris–HCl buffer, pH 7.4, containing 5 mM EDTA and 1 mM dithiothreitol (Tris–HCl buffer) and also [^{3}H]oestradiol-17β (5 nM; [2, 4, 6, 7 $^3H_4$]-

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oestradiol-17β, specific activity 85 Ci/mmole, Radiochemical Centre, Amersham) in the presence and absence of various amounts of diethylstilboestrol or other potential ligands (5 nM–5 μM). After incubation, tubes were maintained at 4°C and an equal vol (400 μl) of charcoal suspension (0.5% (w/v) Norit-A, 0.05% (w/v) Dextran T-70, 0.1% (w/v) gelatin) in Tris–HCl buffer added. The resultant mixture was shaken for 60 min at 4°C and charcoal removed by centrifugation at 800 ***g*** for 10 min. Aliquots (200 μl) of the supernatant containing protein-bound [^{3}H]oestradiol-17β were added to 6 ml scintillation fluid (1 litre toluene, 500 ml Triton X-100, 10 g PPO) and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation.

Determination of nuclear sites by [^{3}H]*oestradiol-17β exchange—'total sites'*

The method for the determination of nuclear oestradiol-17β binding sites is a slight modification of that described in a previous communication [9]. Briefly, receptor protein present in the nuclear fraction (200 μl aliquots) was precipitated by protamine sulphate (1 mg/ml in 50 mM Tris–HCl buffer). [^{3}H]Oestradiol- 17β (20 nM) in Tris–HCl buffer (200 μl) containing 5 nM sodium bisulphite or [^{3}H] oestradiol-17β plus a 1000-fold higher concentration of cold diethylstilboestrol was added to the precipitated receptor and the mixtures incubated for either 4 hr at 15°C or 16 hr at 4°C. After incubation, the tubes were cooled at 4°C and washed repeatedly in Tris–HCl buffer. The final pellet was extracted with ethanol, centrifuged and the ethanolic supernatant counted as before.

Determination of oestrogen binding sites following the in vivo *administration of either oestradiol-17β or various C_{19}-steroids*

(a) Thirty ovariectomised animals bearing 50 mammary tumours were divided randomly into ten groups (5 tumours/group). Each group received either oestradiol-17β (0.5 or 5 μg), testosterone (1 mg), 5α-dihydrotestosterone (1 mg), epitestosterone (1 mg), androstenedione (1 mg), 5α-androstane-3β, 17β-diol (100 μg or 1 mg), or androst-5-ene-3β, 17β-diol (androstenediol) (100 μg or 1 mg) in 1 ml vehicle (steroid solutions were prepared by addition of the requisite compound to propylene glycol and diluting to volume with 0.15M NaCl) by i.v. injection.

Tumour biopsy samples (approx. 200 mg tissue) were removed aseptically from Nembutal®-anaesthetized animals at time 0 (prior to injection) and at 10, 25, 40 and 60 min after injection. Cytosol fractions were assayed for accessible oestradiol-17β binding sites while nuclear fractions were assayed for both total and accessible sites.

(b) Fifteen ovariectomized animals bearing 20 tumours were divided randomly into 4 groups (5 tumours/group). The experimental design was as described in (a) except that animals were treated with either oestradiol-17β (5 μg), 5α-dihydrotestosterone (1 mg), 5α-androstane-3β,17β-diol (1 mg) or androstenediol (1 mg) and tumour tissue was biopsied at time 0, and at 0.5, 1, 2, 4, 8, 16 and 24 hr after injection. Nuclear fractions were assayed for both total and accessible oestradiol-17β binding sites.

Chemical analyses

The DNA content of nuclear fractions and the protein content of cytosol fractions were estimated using the methods of Burton [13] and Lowry, Rosebrough, Farr and Randall [14], respectively.

RESULTS

Specificity of the accessible cytoplasmic oestradiol-17β binding assay

The data presented in Fig. 1 shows the competition by various non-radioactive steroids with [^{3}H]oestradiol-17β for accessible cytoplasmic binding sites. Addition of 25 nM unlabelled oestradiol-17β decreased the binding of [^{3}H]oestradiol-17β to approximately 40% of the control value. The binding continued to decrease with increasing oestradiol-17β concentrations. There appears also to be competition for receptor binding between [^{3}H]-oestradiol-17β and the dihydroxysteroids. Androstenediol and 5α-androstane-3β, 17β-diol in a 1000-fold excess decreased the binding of [^{3}H]oestradiol-17β to 25 and 42% of the control value respectively. Of the other C_{19}-steroids tested, testosterone and 5α-dihydrotestosterone were partially inhibitory but only at very high concentrations, while androstenedione, epitestosterone and dihydroepiandrosterone were without any substantial effect.

In vivo *effects of oestradiol-17β and C_{19}-steroids on* [^{3}H]*oestradiol-17β binding*

Within 40 min following the *in vivo* administration of 0.5 μg oestradiol-17β, an approximately 50% reduction in the binding of [^{3}H]oestradiol-17β to specific cytoplasmic binding proteins was observed (Fig. 2). This corresponds to a concomitant progressive increase in specific nuclear oestradiol-17β binding sites. Elevation of the level of oestradiol-17β adminis-

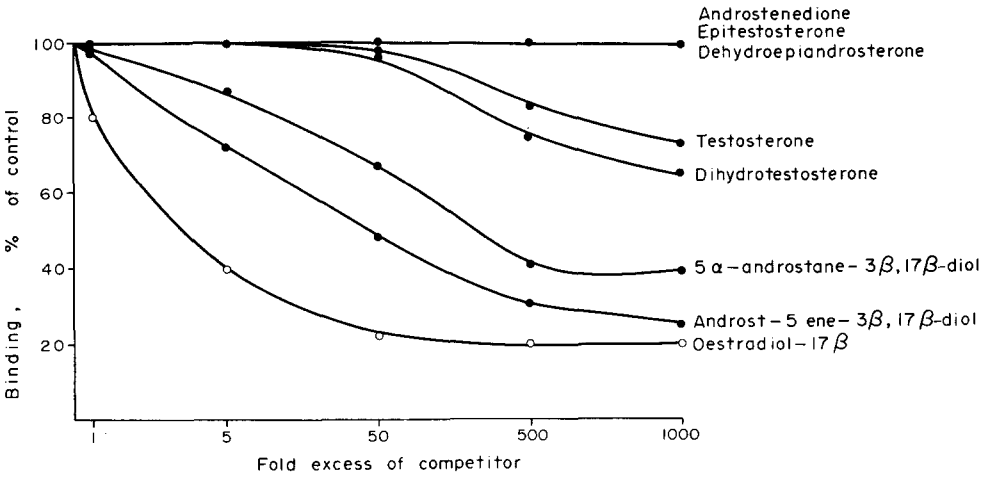

Fig. 1. Specificity of [^{3}H]*oestradiol-17β binding assays. Cytosol fractions were incubated for* 2 hr *at* 4°C *with a saturating concentration of* [^{3}H]*oestradiol-17β* (5 nM) *in the presence or absence of increasing concentrations* (1, 5, 50, 500 *and* 1000*-fold excess) of the potential oestrogen antagonists. After incubation free steroid was removed by charcoal adsorption.*

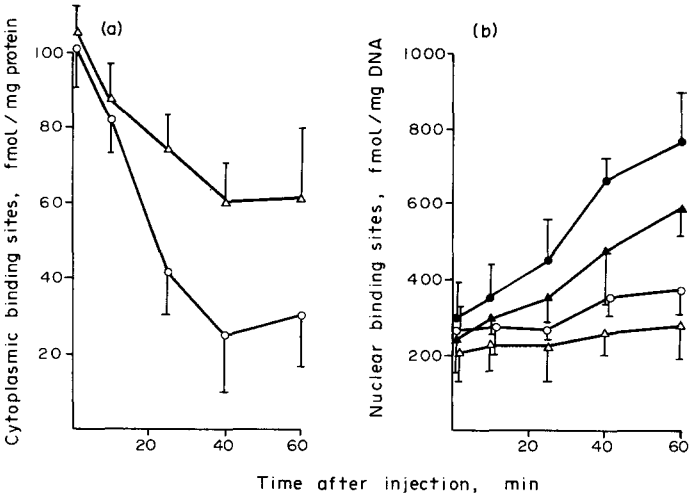

Fig. 2. Effect of in vivo *administration of oestradiol-17β on cytoplasmic and nuclear oestradiol-17β binding sites. Rats were injected with oestradiol-17β* (0.5 μg, △ *or* 5 μg, ○) *at time* 0 *and tumour biopsy samples removed at varying time intervals thereafter. (a) Cytosol preparations from the biopsies were incubated at* 4°C *for* 16 hr *with a saturating concentration of* [^{3}H]*oestradiol-17β* (5 nM) *or* [^{3}H]*oestradiol-17β plus* 1000*-fold excess of stilboestrol. (b) Nuclear fractions were incubated for either* 4 hr *at* 15°C (●, ▲) *or* 16 hr *at* 4°C (○, △) *with a saturating concentration of* [^{3}H]*oestradiol-17β* (20 nM) *or* [^{3}H]*oestradiol-17β plus a* 1000*-fold excess of stilboestrol.*

tered *in vivo* to 5 μg resulted in a more pronounced translocation of cytoplasmic binding sites to the nuclear fraction. Like oestradiol-17β, single injections of either androstenediol (Fig. 3) or 5α-androstane-3β, 17β-diol (Fig. 4) reduced accessible cytoplasmic oestradiol-17β binding sites in a dose-dependent manner. The nuclear binding sites, however, unlike the oestradiol-17β-translocated receptor, appear to be present in a form which is readily accessible to exogenous hormone at 4°C. *In vivo* administration of testosterone resulted in a slight decrease of cytoplasmic binding sites at the higher dose level and a corresponding low nuclear uptake of the receptor complex (Fig. 5). A considerable amount of nuclear binding occurred at 4°C. Administration of dihydrotestosterone was more effective than testosterone at reducing cytoplasmic oestradiol-17β binding sites *in vivo*, but less so than either oestradiol-17β, androstenediol or 5α-androstane-3β,17β-diol (Fig. 5). Again, considerable translocation of the

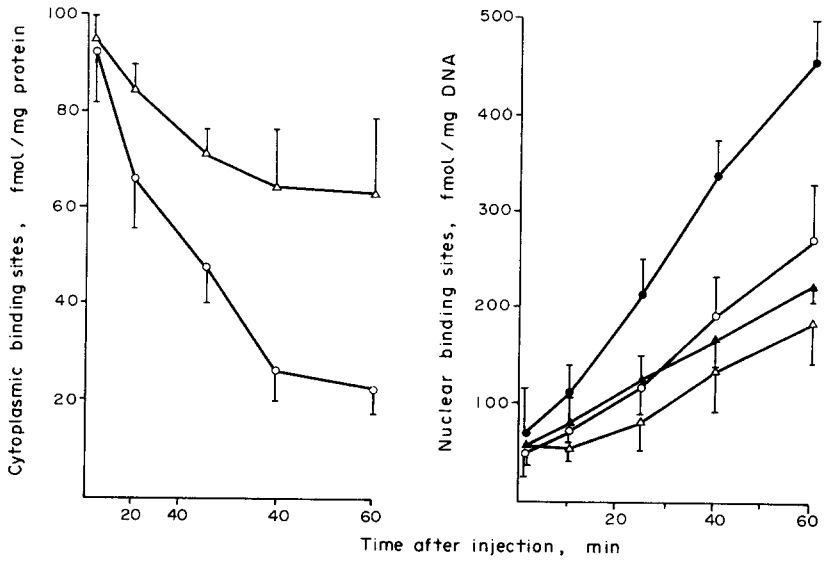

Fig. 3. *Effect of* in vivo *administration of androstenediol on cytoplasmic and nuclear oestradiol-17β binding sites. Details are essentially those in Fig.* 2, *except that the compound administered at two dose levels* (100 μg, △ *or* 1 mg, ○) *was androstenediol.*

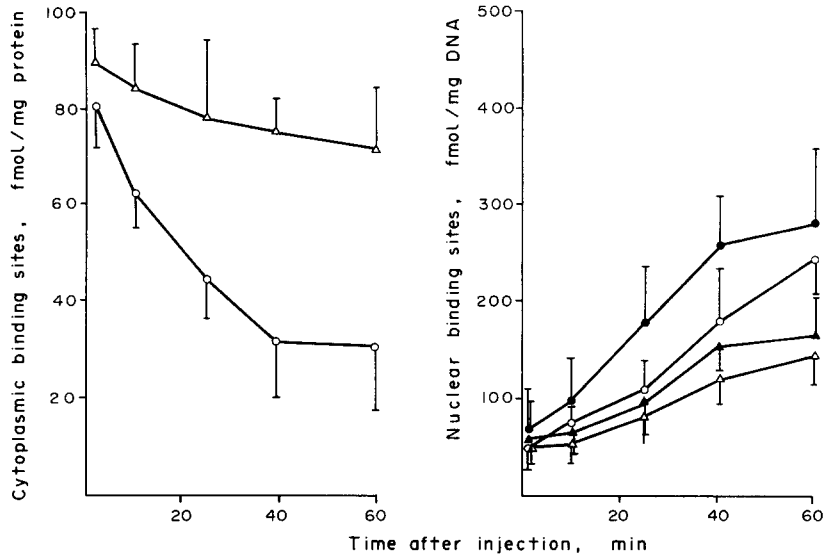

Fig. 4. *Effect of* in vivo *administration of 5α-androstane-3β, 17β-diol on cytoplasmic and nuclear oestradiol-17β binding sites. Details are essentially those in Fig.* 2, *except that the compound administered at two dose levels* (100 μg, △ *or* 1 mg, ○) *was 5α-androstane-3β,17β-diol.*

receptor complex to the nucleus occurred. The receptor bound [^{3}H]oestradiol-17β with equal efficiency at 15 or 4°C. Androstenedione and epitestosterone were without effect on receptor translocation (not illustrated).

Extension of the experimental time period to 24 hr after oestradiol-17β administration (Fig. 6) showed that nuclear binding sites, as determined by exchange assay at 15°C, peak at 2 hr and remain elevated over the 24 hr period. Accessible nuclear binding sites were low throughout the experimental period. The pattern of nuclear binding following androstenediol and 5α-androstane-3β, 17β-diol was substantially different from that produced by oestradiol-17β. Maximum nuclear binding occurred at 2 hr. However, unlike the oestradiol-17β-mediated response, the levels were not maintained over the 24 hr period and returned to values indistinguishable from control levels by 4 hr. 5α-Dihydrotestosterone behaved in a manner similar to androstenediol and 5α-androstane-3β,17β-diol.

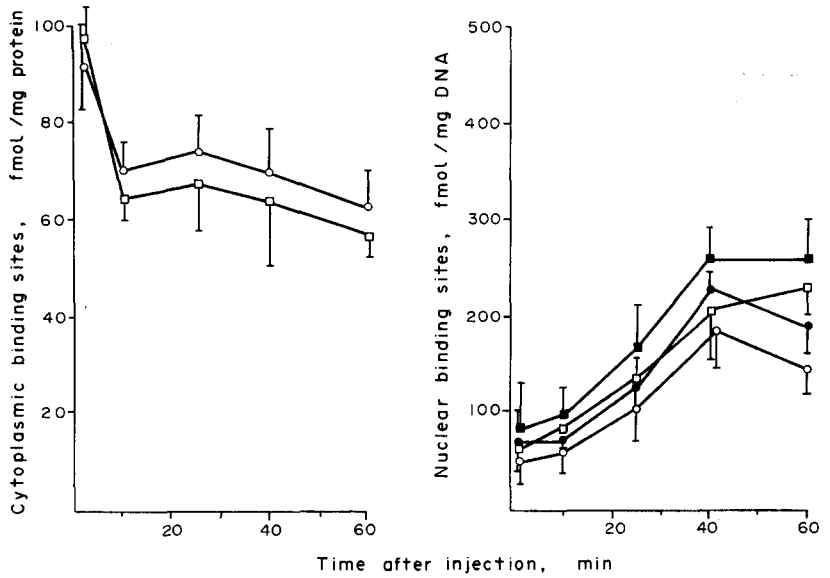

Fig. 5. Effect of in vivo *administration of testosterone and dihydrotestosterone on cytoplasmic and nuclear oestradiol-17β binding sites. Details are essentially those in Fig. 2, except that the compounds administered were testosterone* (1 mg, ○) *and dihydrotestosterone* (1 mg, □).

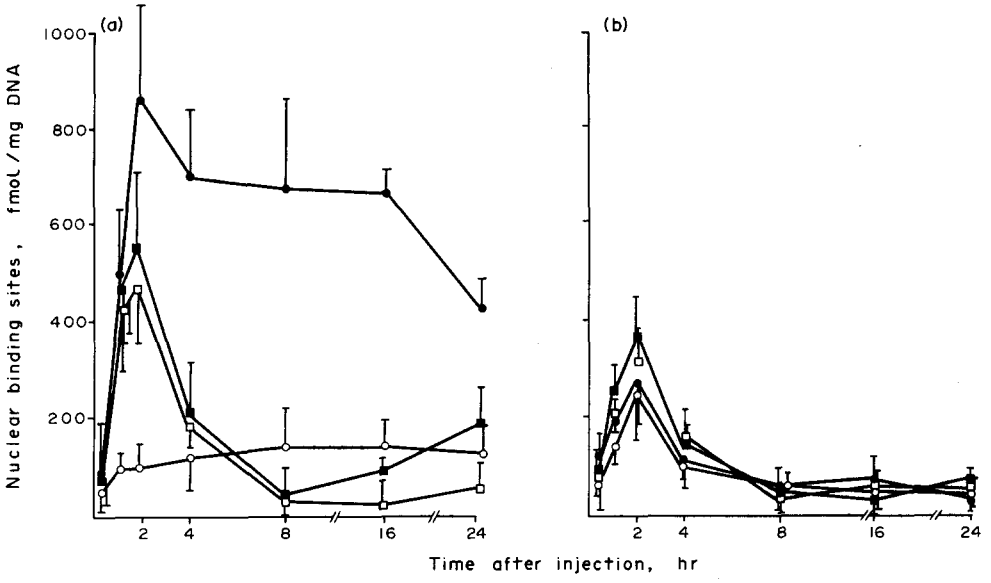

Fig. 6. Effect of in vivo *administration of oestradiol-17β, androstenediol, 5α-androstane-3β,17β-diol and 5α- dihydrotestosterone on nuclear oestradiol-17β binding sites. The experimental design was as in Fig. 2, except that tumour biopsy samples were removed at time* 0, 1, 2, 4, 8, 16 *and* 24 hr *after the administration of steroids. Nuclear fractions were incubated for either* 4 hr *at* 15°C(●, ■) *or* 16 hr *at* 4°C (○, □) *with a saturating concentration of* [^{3}H]*oestradiol-17β in the presence or absence of stilboestrol. The steroids used were (a) oestradiol-17β* (5 μg, ●, ○) *and androstenediol* (1 mg, ■, □) *or (b) dihydrotestosterone* (1 mg, ●, ○) *and 5α-androstane-3β,17β-diol* (1 mg, ■, □).

DISCUSSION

The data presented in Fig. 1 show that androstenediol and 5α-androstane-3β,17β-diol were the most effective compounds examined in reducing the specific binding of [^{3}H]-oestradiol-17β to its cytoplasmic binding protein. Substantial inhibition of [^{3}H]-oestradiol-17β binding was observed at concentrations of these dihydroxysteroids as low as 25 nmole/l, that is at a concentration only 5-fold higher than that of the radioligand. The relative affinity of androstenediol for the oestradiol-17β receptor protein based on comparative binding

studies is approximately 10–20 fold lower than that observed for oestradiol-17β. These observations are consistent with our earlier findings [15] and also with the suggested physiological [15, 16] and pharmacological [10] role of these substances in the growth of mammary tumours.

It is now quite clear that at relatively high dose levels certain androgens can bind to the cytoplasmic oestrogen receptor and induce its translocation to the tumour cell nuclei in a dose-dependent manner. The process appears to be related to the relative affinities of the compounds for the oestradiol-17β receptor protein. Androgens that do not compete with oestradiol-17β for its specific binding protein are unable to affect the translocation process. Similar observations have been made with 5α-dihydrotestosterone in rat uteri by Ruh, Wassilak and Ruh [17] and Rochefort and Garcia [18]. These data are not consistent with specificity previously attributed to the translocation phenomenon [19].

The androgen-transferred receptor is different from the oestrogen receptor complex in both its short nuclear retention time and also in its ability to extensively bind [^{3}H]oestradiol-17β at 4°C. Such data may infer different conformational states for the respective receptors. An analogous finding has been observed with the receptor complex induced by the antioestrogen, tamoxifen, and that formed by oestradiol-17β [20]. Sucrose density gradient centrifugation analysis of rat mammary tumour cytosol fractions reveals that [^{3}H]oestradiol-17β binds specifically with the sedimentation coefficient of 8 S [20–22], while [^{3}H]tamoxifen gives a value of 4–5 S [20]. The exact role or significance of the 4–5 S moiety is unknown. Rochefort, Lignon and Capony [23] demonstrated that the uterine nuclear oestrogen receptor translocated by 5α-dihydrotestosterone bound [^{3}H]oestradiol-17β at 4°C and displayed the characteristic 5 S nuclear oestradiol-17β binding pattern. This latter characteristic does not in itself exclude differences between the respective complexes; rather it may emphasise the relative limitations of the techniques available.

The transitory nuclear retention observed for androgen-transferred oestrogen receptor is similar in character to that observed for oestriol. This steroid apparently acts as a weak oestrogen principally because of its short nuclear retention time [24, 25]. When oestriol is present continually, either by multiple injections [25] or by chemical modification of the compound [26], thus maintaining the nuclear concentration of the receptor complex, it acts as a potent oestrogen without antagonistic properties. In view of these data it may prove valuable to maintain the nuclear concentration of the androgen-transferred oestrogen receptor complex and determine whether it too acts as an oestrogen or has enhanced anti-tumour activity.

In summary, we have shown that certain androgens, most notably androstenediol, translocate the oestrogen receptor to tumour cell nuclei. We propose that this property may form the basis for the known anti-tumour activity of androgens.

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Letter to the Editor

Preservation of Enzymatically Prepared Rabbit VX2 Tumour Cells *In Vitro*

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GALASKO and Haynes [1] have recently reported that suspensions of VX2 carcinoma cells in saline after storage in liquid nitrogen failed to produce tumours in rabbits. However, VX2 tumour cells which had been stored at −70°C in tissue culture medium (TC 199) containing 10% DMSO did give rise to tumours when transplanted into rabbit thigh muscle. The tumour-bearing animals survived longer than the rabbits used for routine passage of tumour. Galasko and Haynes [1] employed a mechanical method for obtaining single cells from the VX2 tumour.

In this communication, we report our experience in preserving the VX2 tumour cells *in vitro*. Initial work with a variety of mechanical techniques for disaggregating the VX2 tumour revealed a viability of only 5–10% in the liberated tumour cells, as determined by trypan blue dye exclusion [2]. Such a low viability suggested that many of the non-staining cells might be damaged to a varying degree. Separation of viable from non-viable VX2 tumour cells was performed by differential flotation on Ficoll–Hypaque density solutions as described by Mavligit *et al.* [3]. The yield of viable VX2 cells recovered from the floating fraction was too poor to render the procedure of practical value.

An enzymatic method as described previously by Muckle and Dickson [4] for disaggregating the VX2 tumour was therefore employed for the present work. In addition to trypsin and deoxyribonuclease, 0.1% collagenase (Type 1 containing protease and peptidase activity, Sigma Chemical Co., St. Louis, U.S.A.) was also added to the enzyme mixture. The viability of the VX2 tumour cells obtained by this method was always greater than 95%. Usually $1–3 \times 10^8$ VX2 cells were obtained from 10 g of tumour. The VX2 cells were washed 2× and resuspended in either Waymouth culture medium (Difco 5043-37-5 TC Medium MB 752/1 Dried) containing 1% bovine serum albumin (BSA: Sigma Chemical Co., Albumin, bovine, fraction V) or in Dulbecco's modified Eagle's medium (MEM: Flow Laboratories, IF-017A, Dried) containing 20% newborn calf serum (Flow Laboratories, 4-053 D). These two media contained penicillin (100 units/ml), streptomycin (100 μg/ml) and Mycostatin (100 units/ml).

The following three methods were employed for storing the VX2 cells.

1. *Storage at room temperature* (*RT*, 20°C)

The VX2 tumour cells were left at RT for 7 days in Waymouth medium containing 1% BSA. The viability of the tumour cells at the end of 7 days was 60–70%. One million viable tumour cells were implanted into the left hind leg muscle of New Zealand white rabbits as described previously [4, 5].

2. *Short-term VX2 cell cultures* (37°C)

The VX2 cell suspensions in MEM + 20% calf serum were seeded into 30 ml (75 cm^2) Falcon flasks (Falcon Plastics, Los Angeles, CA, U.S.A.) using 10 ml suspensions with 1×10^6 cells/ml for each flask. The flasks were incubated at 37°C in an atmosphere of 5% CO_2 in air using a CO_2 incubator. After 24 hr, the cells were put on 50% conditioned medium. The medium was renewed every 2 days using fresh medium after the second day. After 5–6 days, the primary cultures were treated with trypsin–versene (5 ml; 1 vol of 0.25% trypsin + 4 vol of 0.02% versene) and

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passaged—the cells of one flask being split into 2 new flasks as described by Souhami *et al.* [6]. The VX2 carcinoma cells were maintained in culture for up to 6 weeks. One million cells from the short-term cultures were transplanted into rabbits at weekly intervals after initiating the cultures.

3. *Freezing in liquid nitrogen* (−196°C)

The VX2 tumour cells were stored in Waymouth medium containing 1% BSA and 10% glycerol or 10% DMSO. The sealed glass ampules each containing 5×10^6 cells in 1 ml of the above medium were initially placed in a deep freeze at −70°C for 20 hr. Subsequently the ampules were transferred into a liquid nitrogen storage tank at −196°C. When required, the ampules were rapidly thawed in a waterbath at 37°C, and the cells were washed twice in BSA-containing medium followed by BSA-free Waymouth medium. The viability of the VX2 cells preserved in 10% glycerol-containing medium decreased from 95 to 40% after storage of cells in liquid nitrogen for 3 yr. On the other hand, the viability of VX2 cells stored for this period of time in 10% DMSO-containing medium was usually less than 5%. Cell lysis was often observed in the tumour cell suspensions recovered from DMSO-containing medium; this did not occur when glycerol was used as preservative.

Bioassay

The growth characteristics of the tumours obtained in rabbits after inoculation of fresh VX2 cells, or cells stored at RT, in liquid nitrogen and in short-term cultures were similar. From an i.m. inoculation of 1×10^6 viable cells the tumour became palpable by 4 weeks and all animals died with metastases in the lungs, regional and para-aortic lymph nodes at 70 ± 7 days after injection. The VX2 tumour cells grown in culture for over 3 weeks failed to produce tumours when inoculated into rabbits. We have reported the use of such short-term cultures of VX2 tumour cells in microcytotoxicity assays for testing lymphocytes and serum from VX2 tumour-bearing rabbits treated by hyperthermia [7].

The results reported here support the previous findings [1] that VX2 carcinoma cells can be stored *in vitro* and do not necessarily have to be passaged *in vivo*. Galasko and Haynes [1] observed a fibrin clot formation when the VX2 cells were stored at −70°C in TC 199 containing DMSO. Furthermore, successful transplantation of the tumour was ensured only when the fibrin clot was implanted into the thigh muscles of the rabbit. Cells stored for 13 months failed to produce tumours in rabbits [1]. In the present study with the enzymatically prepared VX2 cells, fibrin clot formation was never observed. The VX2 tumour cells stored in liquid nitrogen for up to 3 yr gave rise to tumours on inoculation into rabbits, and glycerol was clearly superior to DMSO as a low temperature preservative for long-term storage of these cancer cells. The greater viability of the initial cell population and the use of storage medium containing albumin as well as glycerol may account for the superior results obtained in the present work.

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Announcement

EUROPEAN ORGANISATION FOR RESEARCH ON TREATMENT OF CANCER

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Perspectives in Cancer Research

Suppressor Cells of Immune Reactivity in Malignancy

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A MAJOR advance in the field of immunology during the past few years has been the concept that immune reactions are controlled by negative feedback mechanisms through suppressor cells [1, 2]. In many immunologic model systems such suppressor cells of various types, particularly suppressor T cells, have been described [3, 4]. There have been a number of reports that suppressor cells may also play a role in the immunosuppression observed in tumor bearers which will be reviewed in the present article.

Immunosuppression plays an important role in many aspects of the cancer problem which may be summarized as follows:

1. According to the immunosurveillance concept a failure of the immune system has been implicated in the development of cancer [5].
2. Specific immune reactivity against tumor cells which has been well documented to exist in many instances [6] appears to be prevented from being operational *in vivo* by immunosuppressive mechanisms [7].
3. Most tumors after they reach a certain size cause a general suppression of the immune system [8]. This suppression is nonspecific in nature but it may well add to the suppression of specific anti-tumor immunity.

SUPPRESSOR CELLS AND IMMUNOSURVEILLANCE

Clinical observations have supported the existence of an immune surveillance mechanism against tumors [9]. A strong case for immune surveillance can be made in the case of virus-induced tumors [10]. However, convincing evidence has been presented that spontaneous tumors are nonantigenic [11]. A concept has been recently offered how to explain this obvious contrast between the nonantigenicity of spontaneous tumors and the role of immunosurveillance against virally induced tumors [10]. Suppressor cells have been described in virally induced tumor systems after the tumors had reached a considerable size [12]. There are no data available supporting the existence of suppressor cells during the early development of virus-induced tumors. Thus, there is at present no indication for a role of suppressor cells in the breakdown of cellular immunosurveillance against virally induced tumors. However, experiments in more suitable models such as in polyoma-virus-induced tumorigenesis have not been performed as yet.

SUPPRESSION OF SPECIFIC ANTI-TUMOR IMMUNITY BY SUPPRESSOR CELLS

There is now a large body of data available to indicate that tumor cells possess antigens which can be recognized by immune lymphocytes of the tumor bearing host *in vitro*. Effector mechanisms of many kinds have been defined but it remains unclear why these are not successfully operative *in vivo* (for review see references [6, 8, 13]). Conceivable, the observed immune reactivity only represents an epiphenomenon which *a priori* has no *in vivo* significance (as for example the immune response to a myocardial infarction [14]). Even if this were so, it would be of importance to study these mechanisms with the goal to alter them for therapeutic use either by modification of the antigenicity of the target cell or by enhancing the activity of the effector cells.

However, it is also possible that the anti-tumor immune response is quite potent, but that it is prevented from being active by blocking mechanisms. This is suggested by the so called "eclipse" phenomenon, observed in certain

experimental tumor systems [15]. In these, anti-tumor cellular immunity can be observed at an early stage of tumor growth, but no longer during progressive tumor growth, presumably due to a tumor-induced immunosuppression. The mechanisms of this immunosuppression are not known. Likewise, little is known about the mechanisms of the suppression of the specific anti-tumor immune response in situations where the eclipse phenomenon does not apply.

Quite extensive evidence has been presented for the involvement of blocking factors, i.e. soluble products capable of interfering with the immune reactivity against tumor cells [7]. Depending on the system studied, these have been suggested to represent antigen, antibody, or antigen–antibody complexes [16–18]. There is beginning evidence to indicate that there is an involvement of suppressor cells in the suppression of the specific anti-tumor immune response. Kirkwood and Gershon have demonstrated suppressor T cells that affect the immune response to an allografted methylcholanthrene-induced sarcoma [19]. Umiel and Trainin [20] and Small and Trainin [21] have shown that in mice bearing the syngeneic Lewis lung tumor suppressor T cells are generated which are able to enhance tumor growth upon transfer into new recipients. In this system, it was also found that adult thymectomy which is known to preferentially remove suppressor T cells, led to a reduction in tumor development. The most convincing evidence for a role of suppressor T cells ablating the host's immune response against a tumor, stems from the work of Fujimoto and his colleagues [22, 23]. These authors have studied mice bearing the syngeneic methylcholanthrene-induced sarcoma 1509a. Solid immunity against a second challenge with this tumor was induced after surgical removal of the primary tumor. Injection of T cells of tumor bearers was found to effectively and specifically interfere with the existing immunity, leading to an inhibition of tumor rejection. Takei and his colleagues have observed that specific killer cells against the syngeneic mastocytoma P815 in DBA mice could be found only at early stages of tumor growth. At later stages specific suppressor T cells were demonstrated which inhibited the cytotoxic activity of killer cells [24, 25].

Thus, in different experimental systems suppressor T cells have been found to interfere with the anti-tumor immune response. These data may be related to Prehn's earlier work which has shown that under certain circumstances immune lymphocytes from tumor bearers have the effect of enhancing tumor cell growth in Winn type assays [26].

SUPPRESSOR CELLS AS A CAUSE OF TUMOR-ASSOCIATED IMMUNOSUPPRESSION

Clinically, many human tumors lead to a depression of cellular immunity [8] and immunosuppression has been also observed in animals bearing experimental tumors [27]. However, in some tumor systems, when the lymphocyte functions measured where not expressed on a per cell basis but per lymphoid organ (such as the spleen which is often enlarged in tumor bearers) it was calculated that these functions were actually increased. It was concluded that the effect of a tumor on the immune system was a stimulatory one [28]. Subsequently, using similar *in vitro* assays, we have brought up a concept which may explain how *stimulation* of the immune system will nevertheless cause *immunosuppression* namely through the activation of suppressor cells [29]. In the meantime similar suppressor cells have been described in many different tumor systems by a number of laboratories [30–40]. These tumors have included a considerable variety of spontaneous and virally and carcinogen-induced tumors of mice and rats. The suppressor cells inhibited the *in vitro* lymphoproliferative reactions to a variety of stimuli. While the suppressor cells were non-specific in nature, tumor-specific immune functions such as the mixed leukocyte tumor cell interaction, or the generation of specific killer cells were also inhibited. The suppressor cells were non-T cells and most investigators now agree that they belong to the monocyte–macrophage series. However, the possibility that they may be B lymphocytes still remains to be ruled out more vigorously.

The occurrence of suppressor macrophages seems not to be restricted to the tumor situation. For example, they have also been found in spleens of mice undergoing a graft vs host reaction or mice injected with *Corynebacterium parvum* [41]. Also, macrophages from untreated rabbits, rats and mice may suppress lymphocyte proliferation [42–45]. Thus, macrophage-mediated suppression of lymphoproliferation may represent quite a general phenomenon which gains particular relevance in the tumor situation. In this context, it has been recently pointed out that the depression of mitogen reactivity by macrophages in spleen cell cultures from tumor bearing rats may be caused only by their increased numbers [40]. This possibility definitively exists, but it also represents a distinct alternative that suppressor macrophages are a particular subclass of macrophages or cells of a state of increased activation.

Yet another type of suppressor cell has been recently described in mice bearing subcutaneously growing plasmocytomas [46]. These also had the characteristics of macrophages and inhibited primary *in vitro* antibody responses to sheep erythrocytes. They had no suppressive effect on mitogen reactivity. Conversely, the suppressor macrophages found in mice bearing murine sarcoma virus-induced tumors which actively suppressed mitogen responses were without effect on the *in vitro* antibody responses to T cell dependent and T cell independent antigens (Kirchner and Chused, unpublished observation). It will be of interest to do parallel tests of both assays using lymphocyte populations from animals bearing the two different tumors.

A suppressor cell has been also described in spleens of mice infected with murine leukemia virus [47, 48]. However, these mice contain leukemia cells in their spleen, which when admixed with lymphocytes have been shown to have marked immunosuppressive effects [49, 50]. The exact nature of the suppressor cell in leukemic spleens remains to be determined.

Suppressor cells of mitogen reactivity have also been found in the blood of owl monkeys [51]. Owl monkeys injected with Herpes virus Saimiri develop a lymphoma which may represent a useful model for lymphoma in man. Suppression of mitogen reactivity of blood lymphocytes was observed even before signs of ouvert leukemia could be detected. The nature of the suppressor cells has not been identified.

The *in vivo* relevance of the suppressor cells of general immunity described above remains to be determined. Unfortunately, there are no approaches available to obtain animals free of macrophages such as neonatally thymectomized or nude mice which are free of T cells. Furthermore, the performance of adoptive transfer experiments is complicated by the fact that macrophages are also functioning as anti-tumor effector cells (e.g. see references [52, 53]. It will be of great importance to determine whether suppressor macrophages and anti-tumor macrophages represent different subpopulations or populations of a different degree of activation or not. Until now it has not been possible to physically separate the cells with the two functions. We are facing the problem that cells with anti-tumor activity are contained in the same fraction as the cells which suppress lymphocyte activity. Such two types cells are found not only in spleens of tumor bearers but also in spleen injected with the immunoadjuvant *C. parvum* widely used in immunotherapy of tumors [41, 54]. The potential significance of such findings for the development of immunotherapy protocols seems to be appealing.

SUPPRESSOR CELLS IN MALIGNANT DISEASE OF MAN

In man, suppressor cells have been found to account for the *in vitro* immunosuppression observed in two lymphoproliferative disorders, Hodgkin's disease and myeloma [55, 56]. More recent reports have demonstrated suppressor cells of lymphoproliferation in patients with a variety of lymphoid tumors [57–60]. In addition, suppressor cells have also been demonstrated in patients with non-neoplastic diseases, such as common variable immunodeficiency [61] and in fungal infections [62]. The latter two cells appeared to be T lymphocytes, while the suppressor cells in the patients with the above-mentioned malignancies, similar to the data in the animal systems, belonged to the monocyte series.

It has been well established that defective humoral immunity is associated with myeloma. Recently, Broder and his associates have found severely depressed responses of peripheral blood lymphocytes from myeloma patients in the *in vitro* assay of pokeweed mitogen-induced immunoglobulin synthesis [55]. They have also found that leukocytes from myeloma patients actively suppressed immunoglobulin synthesis by B lymphocytes from normal individuals. The cell mediating this suppression appeared to be a monocyte. Furthermore, this group has also presented data in a mouse plamocytoma model which supported their previous findings in myeloma patients [63].

In Hodgkin's disease immunosuppression, predominantly of cellular immunity has been a well known clinical feature. Hersh and Oppenheim have shown that the peripheral lymphocytes of patients with Hodgkin's disease were hyporeactive in assays of *in vitro* lymphoproliferation [64]. It was subsequently shown that leukocytes of these patients were also poor *stimulators* in the mixed leukocyte reaction [56]. Removal of adherent cells reconstituted the stimulatory capacity suggesting that the defective stimulation was caused by an adherent suppressor cell. More recently, it was directly shown by Schechter and Soehnlen that monocytes from patients with Hodgkin's disease were suppressing lymphoproliferative reactivity [65].

An initial report has been given as an abstract that in some patients with cancer, the depressed responses of peripheral lymphocytes to mitogens

were caused by suppressor cells [57]. More recently, Blomgren and his associates have shown that the responses of lymphocytes from patients with advanced cancer to PPD-tuberculin and to allogeneic cells were suppressed by suppressor cells [58, 59]. A well documented report of similar results has been presented by Zembala *et al.* [60]. In all the reports evidence for the monocyte/macrophage nature of the suppressor cells was presented. However, in two patients of Zembala's series suppressor T cells were found [60].

CONCLUSIONS

In conclusion, in animal tumor models basically two types of suppressor cells have been described, a suppressor T cell interfering with specific anti-tumor immunity and a non-T suppressor cell, believed by most investigators to be a macrophage, the activity of which may be measured in a number of *in vitro* tests of cellular and humoral immunity. These tests are non-specific in that they don't measure specific anti-tumor immunity. This does, however, not exclude that the nonspecific suppressor cells suppress specific anti-tumor immunity as well.

In the human situation the former suppressor cell will be difficult to detect at present because of the relatively late stage of tumor development at which the investigator has an opportunity to study lymphocyte reactivity and because of the lack of definitive knowledge on the specificities of the anti-tumoral immune response in the human situation [66]. The latter type of suppressor cell has been identified in patients with lymphoproliferative diseases and in patients bearing solid tumors. Probably, when systematic tests will be undertaken, this type of suppression will be found even more frequently. The *in vivo* significance of such suppressor cells remains to be determined. However, since the same is obviously true for the different mechanisms of blocking by soluble mechanisms which have been previously described, further studies of suppressor cells should be encouraged.

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Irradiation of the Lungs as an Adjuvant Therapy in the Treatment of Osteosarcoma of the Limbs

An E.O.R.T.C. Randomized Study

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Abstract—*In* 1970 *a controlled clinical trial was started by the E.O.R.T.C. Radiotherapy Cooperative Group. The aim was to evaluate the effect on the development of lung metastases of an adjuvant irradiation of the lungs after "radical" treatment of primary osteosarcoma of the limbs. Five treatment Centers in France and two in The Netherlands have been participating in this trial. Early* 1975 *the trial was stopped. At that time* 86 *patients had been randomized into a group of* 42 *patients without any adjuvant therapy and a group of* 44 *patients with adjuvant lung irradiation to a dose of* 2000 rad *in* 2 *weeks. The results show that this dose of radiation can significantly decrease the percentage of cases in which metastases develop. This effect is especially seen in the group of patients under* 17 yr *of age, also resulting in a better survival rate for that group. Surprisingly it was found that in one of the participating Centers no beneficial effect from lung irradiation could be demonstrated. No other explanation could be found for this phenomenon than that a selection of patients with unfavourable prognosis is being referred to that Center. The results obtained make it necessary to include, apart from chemotherapy, lung irradiation in future trials on adjuvant therapy in osteosarcoma patients.*

INTRODUCTION

PROGNOSIS for patients with osteosarcoma is very poor. The 5-yr survival rate does not exceed 20–25%. Irrespective of the method of treatment of the primary tumor, prognosis is almost entirely dependent on the appearance of hematogenous metastases. Although metastases may appear in different organs such as skeleton, secondaries in the lungs are the main cause of death. Improvement in prognosis therefore can only be expected through reducing the growth of metastases and particularly of those arising in the lungs. In 1969 it was decided by the clinical cooperative group on radiotherapy of the E.O.R.T.C. to start a controlled clinical trial (O_2) to evaluate the effect of X- or gamma irradiation of the lungs on the percentage of cases in which lung metastases later become demonstrable.

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From data in the literature and from a study by Abbatucci [1] the conclusion was made that a dose of 2000 rad in 2 weeks could be considered as a safe dose with regard to radiation damage of the lungs.

It was decided to apply a dose of 1750 rad in 10 sessions during 12 days, without correction for the lesser absorption in the air-containing lungs. The actual dose to the lung tissue will then be a little less than 2000 rad, when using telecobalt gamma rays or megavoltage X-rays.

Such a dose, assumed to be the maximum permissible one for a "prophylactic treatment" at first sight seems to be inadequate to increase the number of cures. Metastases, detectable on the X-ray of the chest, have a diameter of at least 6–10 mm and contain 10^8 or more tumor cells. Experience has shown that such metastases seldom if ever can be completely destroyed by X-rays even in much higher doses. Apart from the specific radiosensitivity of a certain type of tumor, a chance of cure by radiation is also dependent on the number of viable tumor cells

initially present in the tumor. On the base of radiobiological data it can be demonstrated that for a linear increasing number of equal fractions of X-radiation one can expect an exponential decrease of the number of cells that still can proliferate. When a dose of fractionated X-rays, able to reduce the number of clonogenic cells in the tumor to 10% (D_{10}), is followed by another dose equal to D_{10}, the number of viable cells will be reduced to about 1% and so forth. Though it is improbable that the actual cell survival curve for fractionated radiotherapy will follow such a strict exponential law, it nevertheless may indicate that for a good probability of cure one will need about 11 times D_{10} for a tumour of 10^{10} cells, while for a tumor of 10^4 cells a dose equal to 5 times D_{10} would be sufficient. Assuming a D_{10} of about 400 rad for osteosarcoma, 2000 rad should prevent growth of metastases if the number of tumour cells in the lungs does not exceed 10^4 or 10^5 cells. This might even be higher if local or general defence mechanisms in the body could cope with a small number of viable cells.

To estimate the chances for such a situation in a group of patients with osteosarcoma, Breur reexamined data from earlier measurements on the growth of pulmonary metastases [2, 21].

By extrapolating growth curves backwards in time till the first day of treatment of the primary tumor, it was shown that in the majority of his 13 cases the number of cells present at that time was in all probability already 10^7–10^9, i.e. just invisible on the chest X-rays. In 4 cases, however, the number of tumor cells present in the lungs would have been in the order of 10^5 or less. Thus in theory there seemed to be a possibility for a cure in about 1 out of 4 patients with subclinical metastases at the time of treatment of the primary. Abbatucci [1] also made calculations and came to the same conclusions. It was therefore considered justifiable to start a controlled clinical trial in order to investigate whether radiation of the lungs at the time of initial treatment of the primary tumor would modify the natural course of the disease.

A proposal to irradiate one lung in each patient, using the contralateral lung as a control, was rejected on ethical grounds. The trial was started in January 1970 and was terminated in January 1975.

The following Centres participated:

Institut Gustave Roussy, Villejuif-Paris, France (coordinator: Dr. O. Schweisguth).

Centre Léon-Bérard, Lyon, France (coordinator: Dr. J. P. Gérard).

Centre Françoise Baclesse, Caen, France (coordinator: Dr. J. S. Abbatucci).

Centre Claudius Régaud, Toulouse, France (coordinator: Dr. A. Pons).

Hospices Civils (Centre Hospitalier Régional), Strasbourg, France (coordinator: Dr. J. M. Grunewald).

Rotterdams Radiotherapeutisch Instituut, Rotterdam, The Netherlands (coordinator: Dr. J. G. van Andel).

Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands (coordinator: Dr. K. Breur).

The secretarial tasks for the trial were done in Amsterdam, respectively by Mrs. H. A. van Peperzeel, K. Breur and P. Cohen.

MATERIAL AND METHODS

Selections of patients

Criteria for patients suitable to enter the trial were:

(a) under 60 yr of age;
(b) histologically verified diagnosis of osteosarcoma;
(c) tumour location in one of the extremities;
(d) absence of clinically detectable metastases.

The age limit was laid down to exclude cases of osteosarcoma in patients with Paget's disease of the bones, because of the almost uniformly fatal prognosis for this group.

The histological slides of the tumor tissue, obtained by an open biopsy, were reviewed by R. O. van der Heul, one of the pathologists of the Dutch Bone Tumour Registry. For the diagnosis of osteosarcoma strict criteria were maintained, the main one being the evidence of bone or osteoid formation directly by the tumor cells. Histologically specific types were distinguished like fibrosarcomatous, teleangiatatic types a.o. Also the mitotic activity was noted.

All roentgenograms of the primary tumor as well as the whole lung tomograms were reviewed by one roentgenologist (P. Cohen).

Methods of randomization

Just prior to the planned date for adjuvant lung irradiation, the patient was assigned to one of the two groups:

Group O_2A without adjuvant lung irradiation;
Group O_2B with adjuvant lung irradiation.

This randomization was performed per centre by means of the envelope method, either directly after primary ablative surgery or during the course of the radiation treatment of the primary tumor.

In view of experimental data [3, 4] suggesting an increase in the number of pulmonary metastases after lung irradiation if applied at a time when an actively growing primary tumor is

still present, it was advised to delay the start of the lung irradiation until a dose of at least 4000 rad had been delivered to the primary tumor.

Treatment of the primary tumor

Owing to the fact that in the participating Centers different methods of treatment were applied, no strict rules were given in this respect, as long as such a treatment aimed at a complete local cure. Three main methods of treatment are to be distinguished:

(a) Radiation of the primary tumor to a very high dose (8000–9000 rad) was practised in most French Centers. Ablative surgery was only performed if necessary, i.e. in cases of a local recurrence or complications in the treated area.

(b) Radiation of the primary tumor to a dose of about 6000 rad in 5–6 weeks, followed by an observation period of 4–6 months. After that period ablative surgery was only performed in those cases in which no metastases could clinically be detected. This scheme, which was introduced by S. Cade [5], was practised as a rule in all Dutch Centers.

(c) Immediate primary ablative surgery was performed in a minority of patients (5 in group A, 8 in group B). In two cases (1 in A, 1 in B) the indication was based on histological criteria, suggesting a slightly better prognosis [6, 7]. Furthermore primary surgery was performed in cases with a serious dysfunction of the limb or with a pathological fracture.

Examination of the patient had to include physical examination, roentgenograms of the tumor area, whole lung tomography and blood analysis (blood count, S.R.E., alcaline phosphatase levels). Chest X-rays in two directions and tomography of the lungs had to be repeated just prior to randomization. Isotope scanning of the skeleton and lung function tests were optional.

Lung irradiation

With telecobalt gamma rays or megavoltage X-rays the lungs were irradiated through an anterior and a posterior field with beams encompassing the total contents of the thoracic cavity. Without correction for lesser absorption in the air-containing lungs, a dose of 175 rad was given per session in the mid-plane of the thorax. With 5 fractions per week a total dose of 1750 rad was applied within 12 days. The actual dose delivered to the lung tissue thus has been little less than 2000 rad. This radiation was performed as soon as possible after ablative surgery, or in case of primary irradiation of the bone tumor, when a dose of approximately 4000 rad had been delivered.

Follow-up

Patients were examined from the time of initial treatment at 4- to 6-week intervals during the first year, at 2-month intervals during the second year and 3-month intervals during the third year and later with 4-month intervals. These follow-up investigations included chest X-rays and blood analysis. Other investigations, such as X-rays or isotope scans of the skeleton were only performed on clinical indication.

All data was sent to the secretariat.

Material

When the trial was closed in January 1975, 86 patients had been included, of which 44 patients received lung irradiation. Distribution over the participating centers is shown in Table 1.

Table 1. *Number of patients per center*

Center	O_2A	O_2B	Total
Toulouse	6	3	9
Villejuif	19	18	37
Lyon	5	7	12
Caen	2	0	2
Strasbourg	1	2	3
Rotterdam	5	5	10
Amsterdam	4	9	13
	42	44	86

Of the 120 patients initially registered, 34 had to be excluded before randomization for various reasons. In 4 patients metastases were found just before treatment of the primary tumor. In 15 patients pulmonary metastases could already be demonstrated during the course of radiotherapy of the primary tumor and before randomization. In 4 patients, follow-up data was incomplete, because of emigration. The remaining 11 patients were excluded for different reasons: no confirmation of the histological diagnosis (1 patient); the primary treatment not performed in one of the participating centres (8 patients); patients being over 60 yr of age (1 patient); intercurrent death during treatment of the primary (1 patient).

Sex ratio. The distribution is practically the same in both groups.

In group A: 28 males and 14 females.

In group B: 27 males and 17 females.

The age distribution is presented in Fig. 1, the site of the primary tumors in Table 2. There are no significant differences between the two groups in these respects.

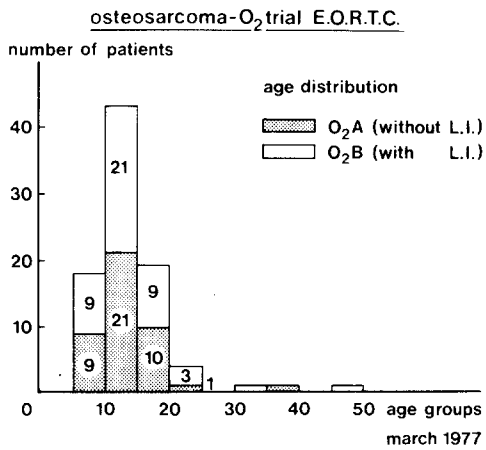

Fig. 1. Distribution of the patients in the O_2 trial over various age groups. The partition is indicated for the groups O_2A without adjuvant lung irradiation (L.I.) aand O_2B with lung irradiation.

Table 2. Site of the primary tumor

	Number of patients		
	O_2A	O_2B	Total
Humerus	3	4	7
Femur	25	29	54
Tibia	13	7	20
Fibula	1	2	3
Radius	—	2	2
	42	44	86

Roentgenology

All X-rays of the primary tumor and of the chest, as well as the tomograms were reviewed by P. Cohen. All tumors showed a more or less typical picture of a malignant bone tumor with characteristics compatible with osteosarcoma, except for one case (group O_2A) in which the X-rays showed a normal cortex and medulla, with periosteal spicules being the only abnormality.

Histology

The diagnosis of all cases included in the trial, was confirmed by the pathologists. It is remarkable that none of the cases had a typical fibrosarcomatous type of tumor. Grading with regard to mitotic frequency and other histologic characteristics did not show any difference between both groups.

Treatment of the primary tumor

As mentioned before three main modalities of treatment of the primary lesion can be distinguished: immediate operation, radiation therapy with delayed amputation (Cade's principle) and high-dose radiation therapy with no surgical procedure planned. The distribution of these treatments over both groups is shown in Table 3.

Table 3. Distribution of patients according to treatment modalities

	Number of patients	
Treatment modality	O_2A*	O_2B*
Direct ablative surgery	5	8
Radiotherapy + delayed surgery ("Cade")	7 (2)	9 (7)
High-dose radiotherapy	30 (13)	27 (10)

*O_2A = without adjuvant lung irradiation.
O_2B = with adjuvant lung irradiation.

Note: The number of patients on which later on amputation was performed is indicated between parentheses.

In 16 patients for whom surgery was planned after radiotherapy 9 patients had no demonstrable lung metastases after the 6 months observation period and underwent an amputation. Of these, 2 patients were in the O_2A group and 7 in the O_2B group. This difference may suggest a delay in appearance of lung metastases in the group with adjuvant lung irradiation. Of 57 French patients who were treated by means of high-dose radiotherapy, 23 had to undergo ablative surgery (13 in O_2A, 10 in O_2B) because of local complications and in a few cases because of renewed tumor activity.

RESULTS

The main criterion for the evaluation of the effect of adjuvant lung irradiation is the percentage of patients without any signs of metastases during the follow-up period.

The periods in which patients could be observed varied from 6 yr to 26 months. The number of patients entered during the successive years is shown in Table 4.

The statistical analysis of the data was done by A. A. M. Hart. The significance tests and the calculations of *P*-values were performed according to the method of Mantel [8]. To compare groups of patients with and without lung irradiation, a one-side test could be used, as it was only of importance to know whether lung irradiation could improve results. To calculate

metastases-free period and survival curves, the product-limit estimator according to Kaplan and Meier [9] was applied. The results presented here reflect the situation in March 1977.

Table 4. Number of cases yearly admitted to the trial

	Year of treatment	Number of patients O_2A	O_2B	Total
	1970	5	4	9
	1971	15	8	23
	1972	8	13	21
	1973	7	8	15
	1974	5	10	15
January	1975	2	1	3
	Total:	42	44	86

The lapse of time during which the two groups of patients are free of metastases is shown in Fig. 2. The difference between the curves is in favour of the group O_2B with adjuvant lung irradiation. The value $P_1 = 0.059$ indicates a significant difference at a 6% level.

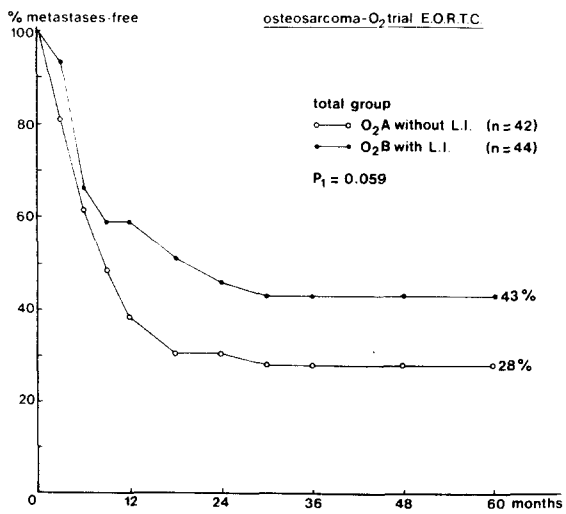

Fig. 2. Actuarial curves for the percentages metastases-free period, respectively for the group O_2A without adjuvant lung irradiation and the group O_2B with adjuvant lung treatment.

The few cases with extrapulmonary metastases are included in the results as all but one of them also developed lung metastases prior to the extrapulmonary metastases.

Both curves remain horizontal after an interval of about 2 yr, indicating an ultimate metastases-free percentage of 43 for the lung irradiated group as compared with 28 in the control group.

Influence of age

The results of adjuvant lung irradiation are relatively better in younger age groups. By breaking down the number of patients in each group into one group above and one under a certain age, it can be shown by the P_1 values (Fig. 3) that for patients under 17 yr of age, the

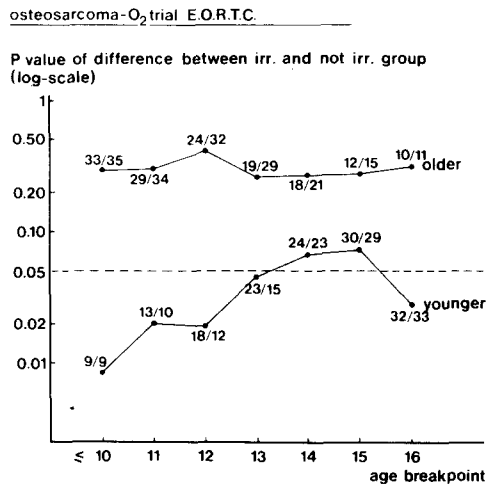

Fig. 3. P_1 values for the difference with regard metastases-free period between the groups of patients without or with adjuvant lung irradiation. At each age the total number in each group is divided into the number of patients of that age or younger (placed on the lower line) and the number of patients above that age (placed on the upper line).

Example: For the age breakpoint 16: there were 32 patients in the non-irradiated group and 33 in the lung irradiated group with an age of 16 or younger; respectively 10 and 11 with an age above 16 yr. For most of the breakdowns the younger age groups show a significant increase in metastases-free period by adjuvant lung irradiation.

difference in favor of adjuvant irradiation remains statistically significant in most of the breakdowns. On the other hand it can also illustrate that for the older age groups, the difference is insignificant.

Looking at the group under 17 yr of age (16 yr and younger), being the group seen in children's hospitals, the curves in Fig. 4 show a postpone-

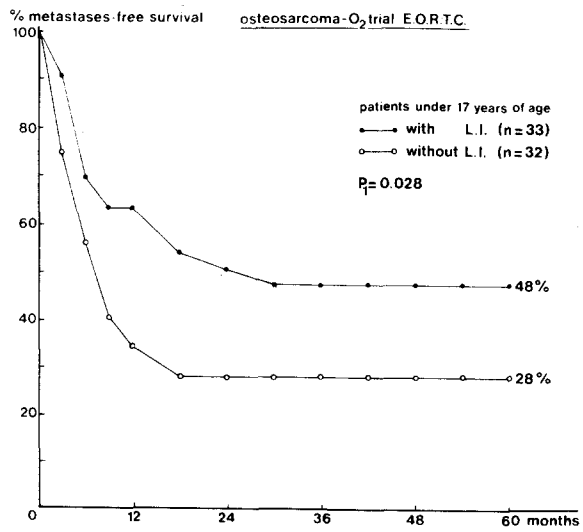

Fig. 4. Patients under 17 yr of age (16 yr and younger). Acturial curves for the percentages metastases-free period, respectively for the group O_2A without adjuvant lung irradiation (L.I.) and the group O_2B with adjuvant lung treatment.

ment of the appearance of pulmonary metastases in the lung irradiated group and a constant percentage of patients remaining free of metastases after 3 yr of 48% compared with 28% in the control group. This indicates that the applied dose of irradiation to the lungs can permanently prevent growth of subclinical metastases in about 28% of the cases. The difference between the curves is statistically significant. ($P_1 = 0.028$).

Influence of treatment modality for primary tumor

In Table 5 the actually observed number of patients who remain free of metastases during observation periods of more than 2 yr (up to 6 yr) are presented. A comparison of results between the groups O_2A and O_2B is not possible for the small groups of patients that underwent direct ablative surgery. Moreover, as mentioned earlier, this intervention almost always was carried out on a special indication, either for bulky tumors, fractures, or in a few cases for an histology suggesting a favorable prognosis. The results of primary radiotherapy with planned surgery (Cade principle) and those of primary high dose radiotherapy (with amputation only if necessary) do not differ significantly. There proves to be a striking resemblance between the chances for metastases-free survival indicated by the actuarial curves (Fig. 2) and the actually observed percentage for the total number of patients in both groups after more than two years observation.

Results according to treatment Centers

The great contribution of patients by the Institut Gustave-Roussy (Villejuif, Paris) made it possible to compare this group of 38 patients with the group of 48 patients treated by the other participating centers. This comparison revealed remarkable differences (Fig. 5). The metastases-free period was markedly shorter for the Paris Group than for the others. The ultimate metastases-free percentage for patients treated in Paris is 33% in the adjuvant treatment group, which is hardly different from the 27% found in the control group. For the patients in the other Centers the percentages are 50% in the treated group and 30% in the controls; this is a

Table 5. Actually observed number of patients remaining free of lung metastases during a follow-up of more than 2 yr

A. Treatment modality	O_2A*	O_2B*
Direct ablative surgery	3/5	2/9
Radiotherapy + delayed surgery ("Cade")	2/7 (28.5%)	4/9 (44%)
High-dose radiotherapy	7/30 (23%)	13/26 (50%)
B. Treatment centers		
Paris	5/19 (26%)	6/18 (33%)
Other centers	7/23 (30%)	13/26 (50%)
C. Age		
<17 yr	9/32 (28%)	16/33(48.5%)
17 yr and older	3/10 (33%)	3/11 (36%)
Total:	12/42 (28.5%)	19/44(43%)

*O_2A = without adjuvant lung irradiation.
O_2B = with adjuvant lung irradiation.

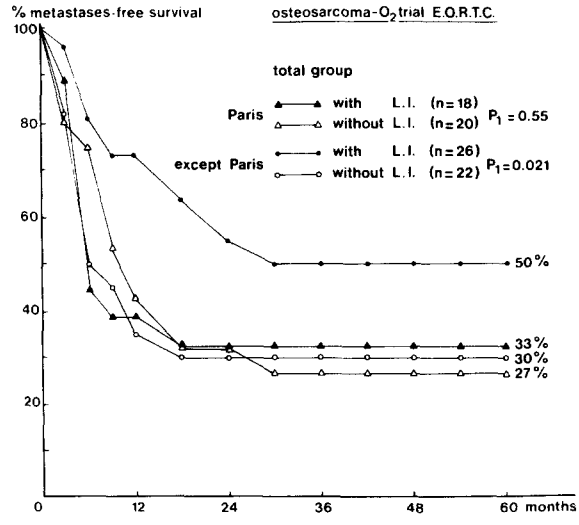

Fig. 5. Percentage metastases-free survival curves for patients treated in the Institut Gustave-Roussy ("Paris") and the combined group of patients treated in the other centers ("except Paris"), comparing the groups with or without lung irradiation (L.I.).

statistically significant difference ($P_1 = 0.021$).

The difference between Paris and the other Centers with respect to the effect of elective radiotherapy on the metastatic free interval still might be due to chance alone ($P = 0.12$). However, tests for these kind of differences—in this case an analogue of the test of Zelen [10] based on the results of the Mantel test on the effect of elective radiotherapy in the two groups (viz. Paris and other centres)—are rather insensitive, making a non-significant result not very conclusive.

The same shift in results could be observed for the patients under 17 yr of age (Fig. 6); practically no difference between the groups for the Paris patients and a highly significant one for patients in the other Centers, of which the lung irradiated group remains free of lung metastases in about 60% of the cases.

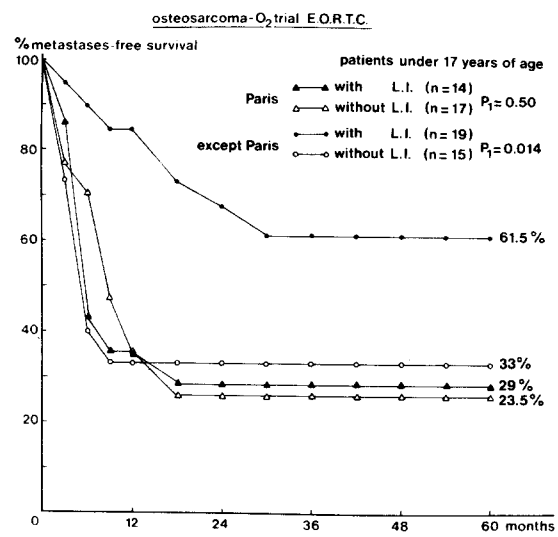

Fig. 6. Patients under 17 yr of age (16 yr or younger). Percentage metastases-free survival curves for patients treated in the Institut Gustave-Roussy ("Paris") and the combined group of patients treated in the other centers ("except Paris"), comparing the groups with or without lung irradiation (L.I.).

In order to find an explanation for the deviating results obtained in Paris, all available criteria have been examined. The criteria for admission and randomization procedure were reexamined and proved to have been followed conscientiously. No apparent differences could be found between the group of patients treated in Paris and those in the other centers with regard to the distribution in site, size, histology or radiographical characteristics of the primary tumor. The average time interval between the first symptom and the first treatment does not seem to have been longer in Paris than in the other Centers. The primary treatment in Paris, as in all other French Centers, has been a high-dose radiotherapy. Since in the Dutch centers the primary tumor was also irradiated primarily (Cade scheme) in most cases and only a minority of cases had direct ablative surgery, the differences in results cannot easily be explained by the method of primary treatment (see also Table 5).

One marked difference, however, was that in 12 out of 49 patients initially registered in Paris lung metastases became demonstrable already during the first weeks of treatment of the primary tumor, while in the other Centers this was only 3 out of 52 patients. Obviously the percentage of patients with metastases in just subvisual size at the time of the first treatment is larger in Paris than in the other Centers.

Extrapulmonary metastases

Skeletal metastases were observed in 12 patients (14%), of which 4 in group O_2A and 8 in group O_2B. In all but one case pulmonary metastases were also present, which makes the presented data also valuable for the criterion disease-free period.

Survival

After appearance of metastases the clinicians were free in their choice of further treatment. In many cases some form of chemotherapy has been applied. In a small number of cases surgical removal of lung metastases was performed: 5 in group O_2A and 3 in group O_2B. This proved to have been successful in 4 cases, respectively 2 in group O_2A and 2 in group O_2B. Because of these treatments the survival curves have to be interpreted with reserve. A longer observation time is needed and probably will show differences more clearly.

For the total groups (Fig. 7) the curves end up with a difference of 15% in favour of the

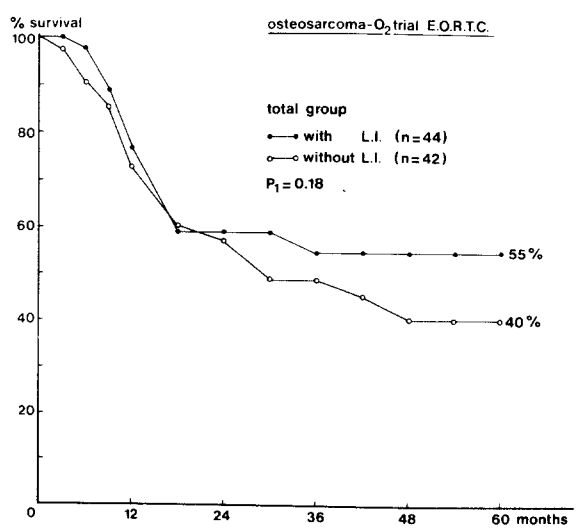

Fig. 7. Actuarial percentage survival curves for the groups of patients with or without lung irradiation (L.I.).

adjuvant treatment, but this difference is not statistically significant. For the patients under 17 yr of age (Fig. 8) the difference after 4 yr between the treated group (59%) and the controls (35%) is almost statistically significant ($P_1 = 0.052$). Comparison of the survival curves for the patients under 17 yr in Paris and in the other centres (Fig. 9), again indicates a different

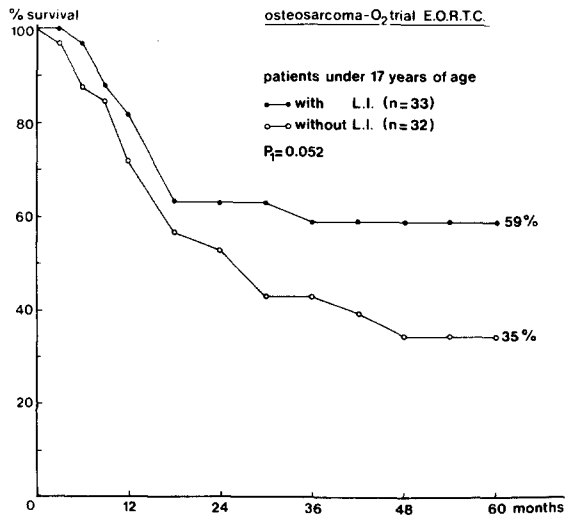

Fig. 8. Patients of 16 yr *of age or younger. Actuarial percentage survival curves for the groups of patients with or without lung irradiation (L.I.).*

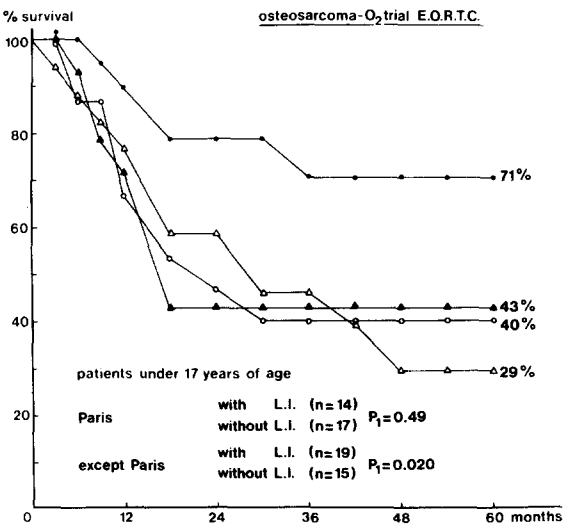

Fig. 9. Patients of 16 yr *of age or younger. Actuarial percentage survival curves for the groups of patients with or without lung irradiation (L.I.). Presented separately for patients treated in the Institut Gustave Roussy ("Paris") and those treated in the other centers ("except Paris").*

effect for the Paris patients. The percentage of surviving for more than 3 yr for patients in the Centers outside Paris reaches the high level of 71% suggesting a possibility of adjuvant lung irradiation to reduce the number of deaths in this age group by half.

The survival curves after the appearance of metastases (Fig. 10) are identical for the treated group and the controls which suggests the absence of a differential effect of the treatment for metastases between the groups.

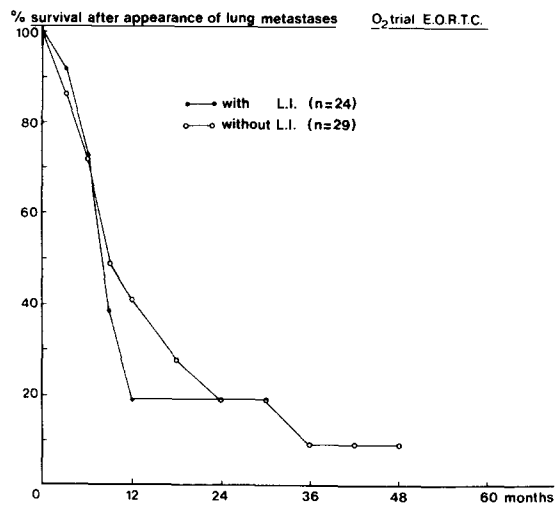

Fig. 10. Survival after the date on which lung metastases were clinically detected. There is no difference in the actuarial percentual survival curves for the groups of patients with or without adjuvant lung irradiation (L.I.).

Complications

The adjuvant lung irradiation of 2000 rad in 2 weeks has caused no symptoms of impairment of the lung function in any of the patients. As the performance of lung function tests was optional, a regular check of the lung function during follow-up has been done only in a small number of cases, in which up till now no decrease in function has been found. [11] No skeletal deformities or growth retardation have been reported.

DISCUSSION

At the start of the E.O.R.T.C. trial early in 1970 no data was available in literature on the effect of any form of adjuvant therapy following treatment of osteosarcoma.

In 1972 Newton [12] published his results of elective lung irradiation in patients with osteosarcoma. Compared to other published data the proportion of the patients in whom lung metastases could be demonstrated after 11 months seemed less (4 out of 11) in the lung irradiated group. Later the results proved to be less convincing. Recently Rab *et al.* [13] published their results of a study on 53 patients with osteosarcoma of whom 26 received whole lung irradiation to a total dose of 1500 rad combined with oxygen breathing and actinomycin D. Randomized by consecutive order of registration the other group of 26 cases did not receive any

adjuvant therapy. No significant difference in survival was found between the groups. In the adjuvant treated group no detrimental effects on the lungs were observed clinically.

During the past 5 yr several reports have been published on the initial results of various schemes of chemotherapy as adjuvant treatment in osteosarcomas [14–16]. Most of these showed a marked postponement of the appearance of metastases, suggesting the possibility of an increase in final cure rate. Unfortunately these trials were not performed as randomized studies, but data from historical series have been used for comparison. That such a method can hardly provide a conclusive evidence may be illustrated by the data presented by Taylor *et al.* [17] showing a marked increase in percentage survival during the latter years without any adjuvant therapy. Most of the published results on adjuvant chemotherapy were only initial in view of the rather short observation periods after the end of the treatment courses, being one to two years after treatment of the primary. For some of the series a few patients were even still under treatment at the time of evaluation of results. Since, it has become evident that in an appreciable number of cases metastases start to appear after stopping the administration of the chemotherapeutic agents. Still these rather premature reports unfortunately made the E.O.R.T.C. group decide to stop entering new patients in the trial early 1975, in order to enable the clinicians to explore the possibility of chemotherapy. Thus the number of patients in each arm of the trial remained rather low. In contrast to most of the authors on adjuvant chemotherapy, our clinical cooperative group decided not to publish results before a sufficiently long follow-up period for the majority of the patients had been reached. This in order not to evoke unjustifiable optimism.

The results of the E.O.R.T.C. trial as presented here, show that 2000 rad of supervoltage X-rays, administered in 2 weeks to the lungs, can permanently suppress the growth of subclinical metastases. This effect is the more striking in the group of patients under 17 yr of age. For this group also a significant increase in overall survival can be expected.

A disconcerting result of the analysis is the finding that the adjuvant irradiation did not improve results in a convincing way for the group of patients treated in the Paris Center. The available characteristics of the group of patients did not provide for an explanation. It has to be accepted that a different selection of patients is referred to this Center, resulting in less effect of the lung irradiation.

It may be assumed that metastases becoming clinically manifest only after a long interval, did contain fewer tumor cells at the time of treatment for the primary as those appearing shortly after the first treatment. Several of the metastases-free survival analysis show a diverging with time of the curves for the groups with and without lung irradiation. This means a larger growth delay period for the later appearing metastases and a greater radiosensitivity of the very small tumor deposits.

The hypothesis that by suppressing the growth of lung metastases extrapulmonary metastases could develop and become clinically detectable in a higher proportion of the cases, has not been confirmed. It was found that 14 from the total group of 86 patients have developed skeletal metastases. This proportion (14%) is within the usual range for other series.

The fact that 8 occurred in the lung irradiated group and 4 in the control group, does not have any real significance, as the cases in the irradiated group had lung metastases at the same time as well.

During the past 5 yr it could be shown that surgical removal of lung metastases of osteosarcoma can result in a definite cure of the patient. Especially in cases where the number of metastases is low and the appearance was more than half a year after the primary treatment or when the growth rate was lower than a volume doubling time of 40 days, the chances for success of metastatectomy are considerable [18–20].

At this moment it remains very difficult to describe an optimal treatment for patients with osteosarcoma. As adjuvant chemotherapy several combinations of drugs have been proposed in various schedules, spread over periods up to 2 yr. In contrast to the 10 sessions of lung irradiation in two weeks, that are tolerated without any difficulty to the patients, the burden of most of the chemotherapy courses to the patients is considerable and sometimes even unbearable. A few fatal casualties and some serious complications during chemotherapy have been reported [14]. Late effects of such intensive therapy are as yet not fully known. In view of all this and the fact that about 20–30% of the patients do not need this adjuvant therapy, it is the more distressing that a reliable estimate of the ultimate improvement in survival still cannot be given.

As some centers cannot confirm the extremely good results reported in the literature, the long-term value of adjuvant chemotherapy still has to be demonstrated in reliable controlled studies. The results obtained in the O_2 trial (E.O.R.T.C.) do make it inevitable to include a

comparison between adjuvant chemotherapy and adjuvant lung irradiation in new trials to be undertaken. Also the possibilities of a combination of lung irradiation and chemotherapy should be seriously investigated. The clinical cooperative group on radio- and chemotherapy of the E.O.R.T.C. is organizing a pilot study on the feasibility of such combination with regard to lung tolerance. A new trial on adjuvant therapy in osteosarcomas is also being prepared by the group. Cooperation of many centres will be needed to guarantee a sufficient accrual rate of patients in such a trial.

CONCLUSIONS

1. A dose of approximately 2000 rad megavoltage or telecobalt radiation to the lungs is able to reduce the probability of clinically detectable lung metastases in patients treated for osteosarcoma.
2. This effect is even more convincing in patients under the age of 17.
3. The results in one of the participating Centers (Paris) are different from those in the other Centers. In Paris the lung irradiation had no significant effect. It seems likely that at the time of adjuvant lung irradiation the occult metastases in Paris were larger on the average than those in the other Centers.
4. Such differences in results between centers can only be detected in cooperative trials in which many centers participate. Exceptionally good or bad results from one center therefore can be misleading as a guide for therapy.
5. In view of the results of the E.O.R.T.C. trial lung irradiation should be included in future clinical trials.

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Effect of Tamoxifen Pre-Treatment on the Retention of Tritiated Oestradiol and 5α-Dihydrotestosterone and on Glucose Metabolism in Human Breast Carcinomas

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Abstract—*The effect of pre-treatment with tamoxifen glucose metabolism and retention of injected oestradiol-17β and 5α-dihydrotestosterone by human breast carcinomas were studied in patients undergoing mastectomy. The following effects were observed:* 1. *The pretreatment reduced retention of oestradiol-17β whereas a small but statistically significant rise in 5α-dihydrotestosterone accumulation was observed.* 2. *There was an increase in both phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase (G6PDH) activities in tumours from treated patients whereas α-glycerolphosphate dehydrogenase (α-GPDH) activity was significantly reduced in the same tumours.* 3. *The significance of these findings is discussed and it is argued that these changes in carbohydrate metabolism may not be due to the blocking of hormone receptors.*

INTRODUCTION

TAMOXIFEN (ICI 46474) is classed as an anti-oestrogenic compound in some species [1] and this property of the drug is considered to be the main reason for its successful use in the treatment of patients with advanced breast cancer [2–4]. Earlier studies on mammary tumours indicate that the effect is probably due to competition with oestradiol for binding at target sites [5–7] but recent investigations on breast cancer patients and on oligospermic men suggest that the drug can induce major alterations in the endocrine environment [4, 8]. It is known that human breast tumours have the ability to retain *in vivo* or bind *in vitro* oestradiol-17β and 5α-dihydrotestosterone (5α-DHT) [9–12] and, therefore, if the action of the drug on the neoplasm is mediated via the depletion of cytoplasmic receptors, there should be alterations in retention of the hormones. On the other hand, if the drug affects the tumour indirectly by altering the endocrine environment, then this may be reflected in other biochemical processes. It is known that both oestrogens and androgens induce changes in the levels of enzymes associated with carbohydrate metabolism in hormone-dependent tissues [13, 14]. Therefore, it was decided to investigate the effect of tamoxifen treatment on the retention of oestradiol and 5α-DHT and on the changes in the activities of some of the enzymes associated with glucose metabolism. This paper reports our findings.

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MATERIAL AND METHODS

All the chemicals used in the study were purchased from British Drug Houses Ltd. and were of analytical grade. α-GPDH, TPI, G6PDH, aldolase, NADH, $NADP^+$, ATP, F-6-P, G-6-P, 6PG and pyruvate were purchased from Boehringer Mannheim. G-1-P was purchased from Koch-Light Ltd. DHAP was prepared from the dicyclohexylammonium salt of the dimethylketal according to the manufacturer's (Boehringer Mannheim)

instructions.* [6, 7-^{3}H]-oestradiol (Sp. act. 56 Ci/mmole), [4-^{14}C]-oestradiol (Sp. act 31.8 mCi/mmole), [1α, 2α(n)-^{3}H]-5α-dihydrotestosterone (Sp. act 55 Ci/mmole) and [4-^{14}C]-5α-dihydrotestosterone (Sp. act. 56.1 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England and were purified by paper chromatography before use. The maker's specific activities were not checked. The tritiated compounds were then dissolved in ethanol to a concentration of 50 μCi/ml. oestradiol and 20 μCi/ml. 5α-DHT. Tamoxifen was purchased from Imperial Chemical Industries.

The patients in these investigations were women with breast carcinoma, undergoing mastectomy, whose informed consent was obtained prior to treatment. All the patients were admitted to the hospital three days before the operation and those who underwent tamoxifen treatment prior to mastectomy were immediately put on the regime. The drug was administered orally (50 mg twice a day) for three days. Just before the operation the tritiated hormone was diluted with 20 ml of 0.9% NaCl and injected into an antecubital vein. The malignancy of the neoplasm was confirmed prior to the operation by drill biopsy. After the mastectomy the tumour was removed, cut into two pieces, frozen immediately on solid carbon dioxide and remained in that state until further processing.

One piece of tumour was thawed, cleared of the surrounding fat or connective tissue, weighed and cut into small pieces. The radioactive compounds from the tissue were extracted and isolated according to the procedures described previously [9, 12, 15–17]. The results are expressed as mean ±S.D. and the differences in retention of tritiated hormones were tested for significance by a Student's "*t*"-test.

Measurement of enzyme activities in breast tumours

The second piece of tumour was used for the measurement of PFK G6PDH, 6PGDH, PGM, LDH, α-GPDH and PHI activities according to the procedure of Shonk and Boxer [18] as modified by Deshpande, Mitchell and Millis [19]. Deoxyribonucleic acid (DNA) was measured according to the procedure of Burton [20]. The results were calculated on the basis of units (U) of enzyme activity per gram wet weight of tissue or milligram of DNA. A unit is defined as that amount of enzyme which will catalyse the transformation of 1 μmole of substrate per min. The results were analysed in two ways: as units of enzyme activity per gram wet weight of tissue or per mg of DNA. They are presented as mean ±S.D. and were tested for significance by a Student's "*t*"-test.

RESULTS

The activities of seven enzymes associated with glucose metabolism in tumours from 12 patients treated with tamoxifen and from 29 control patients are shown in Table 1.

The activities of PFK and G6PDH were significantly raised in neoplasms from treated patients. α-GPDH activities were not different when expressed in terms of gram wet weight but were significantly low in treated cases when expressed on the basis of DNA measurements.

The retention of 5α-DHT and oestradiol-17β after a single intravenous injection of 20 and 50 μCi of tritiated compounds, respectively, is shown in Table 2.

There was a statistically significant increase in the retention of 5α-DHT in the neoplasms of patients who underwent the tamoxifen treatment prior to mastectomy. The retention of oestradiol-17β was significantly lower in tumours from treated cases than in neoplasms from control cases.

DISCUSSION

Most research on the mechanism of action of antioestrogenic compounds is directed towards their ability to inhibit oestrogen induced growth in rat mammary tumours or uterus [21–23]. It has been reported that, like oestradiol, tamoxifen induces early effects such as: depletion of cytoplasmic receptor, concomitant increase in nuclear receptor, subsequent replenishment of cytoplasmic receptor and increases in RNA polymerases B and A activities. Unlike oestradiol, it fails to maintain these changes for a longer period [24]. Furthermore, long term administration of the drug to recurrent breast cancer patients also alters both pituitary and ovarian function [4]. Thus, in any clinical investigation the differences observed in treated cases and controls could be due to direct competitive

*The following abbreviations are used in this paper. *Enzymes:* α-GPDH, α-glycerolphosphate dehydrogenase; TPI, triosephosphate isomerase; G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; 6PGDH, 6-phosphogluconate dehydrogenase; PGM, phosphoglucomutase; LDH, lactate dehydrogenase; PHI, phosphohexose isomerase.
Co-enzymes: NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.
Substrates: F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; 6PG, 6-phosphogluconate; G-1-P, glucose-1-phosphate; DHAP, dihydroxyacetone phosphate.

Table 1. Enzyme activities (μmole of pyridine nucleotide reduced or oxidised/min/gram tissue or mg DNA) in breast tumours from 12 patients treated with tamoxifen (50 mg, twice daily) for three days prior to mastectomy and in 29 untreated tumours

	Treated		Untreated	
	U/g	U/mg DNA	U/g	U/mg DNA
PFK	0.203 ± 0.124*	0.090 ± 0.044	0.067 ± 0.040	0.033 ± 0.019*
G6PDH	0.313 ± 0.233*	0.163 ± 0.107	0.186 ± 0.117	0.077 ± 0.042*
6PGDH	0.155 ± 0.064	0.086 ± 0.039	0.136 ± 0.066	0.084 ± 0.048
PGM	1.893 ± 0.655	0.961 ± 0.380	1.480 ± 0.717	0.619 ± 0.260
LDH	9.127 ± 4.332	5.063 ± 2.566	7.087 ± 3.411	3.820 ± 1.930
αGPDH	0.215 ± 0.180	0.065 ± 0.047	0.196 ± 0.137	0.168 ± 0.116†
PHI	9.085 ± 3.626	5.271 ± 2.708	7.303 ± 3.420	4.955 ± 2.481

The results are expressed as mean ± S.D.

* Significant differences between treated and untreated tumours $P<0.001$.

† Significant differences between treated and untreated tumours $P<0.01$.

Table 2. Accumulations of tritiated 5α-dihydrotestosterone and oestradiol-17β by human breast tumours after a single i.v. injection of 20 and 50 μCi of the hormone respectively

	5α-Dihydrotestosterone		Oestradiol-17β	
	Untreated	Treated	Untreated	Treated
Time interval in min	159 ± 80	180 ± 56	250 ± 90	130 ± 40
Hormone retained dis/min/g	400 ± 120(8)	590 ± 180†(6)	2170 ± 970*(7)	990 ± 220(7)

Time interval represents interval between the injection of hormone and removal of the breast. The results were calculated as disintegrations/minute/gram wet weight and are expressed as mean ± S.D. Numbers in parentheses indicate number of tumours in each category.

* Significant differences between treated and untreated patients. $P<0.01$.

† Significant differences between treated and untreated patients. $0.02<P<0.05$.

action of the drug on the tumour, indirect effect through an altered endocrine environment or a combination of both these factors. Administration of the drug to DMBA-treated rats has resulted in the reduced retention of radioactive oestradiol *in vivo* or lower binding to cytoplasmic receptors in mammary tumours *in vitro* [22]. A similar phenomenon is reported for human breast tumour cells in continuous tissue culture [6]. Therefore, it is reasonable to assume that the reduction in the uptake of radioactive oestradiol reported here in tumours from tamoxifen treated patients is probably due to the occupation of available binding sites by the drug. The other possibility, that the reduction in uptake is due to changes in prolactin secretion, may be discounted since plasma prolactin levels in DMBA-treated rats [23] or in breast cancer patients [4] did not alter after the administration of the drug.

The role of androgens in human breast cancer is not clearly understood. About one fifth of the patients with metastatic disease respond to treatment with androgens [25] and similar responses are seen in rats bearing DMBA-induced mammary tumours [26, 27]. Specific receptors for 5α-DHT have been identified in human breast tumours [10, 11] and the ability of these neoplasms to retain the hormone has been recorded [12]. Our preliminary results on a small series of patients indicate that pre-treatment with tamoxifen slightly increases the uptake of radioactive 5α-DHT. However, further studies are needed to confirm these findings and to investigate the mechanism of action of the drug on androgen receptors.

If the growth of human breast tumours is dependent on oestrogenic stimuli, then the effect(s) may be mediated via alterations in energy generation in the cells. Since the cell's

energy requirements are largely satisfied by carbohydrate metabolism, the measurement of selected enzyme activities in neoplasms of patients treated with tamoxifen and in controls may give insight into the hormonal regulation of glycolysis. Therefore, we have measured the activities of certain key enzymes in breast tumours and observed that the activities of 6PGDH, PGM, LDH and PHI were similar in both treated and untreated patients which suggests that the drug does not affect either glycogen metabolism or lactate production. The tumours from treated patients showed significantly higher activities of PFK and G6PDH and lower activity of α-GPDH. Since PFK represents the first regulatory step unique to the glycolytic pathway, an increase in its activity together with unchanged lactate production indicates that either pyruvate or other intermediary substrates are channelled into other metabolic pathways. Increase in G6PDH activity could be a way of providing reduced NADP and/or precursors for nucleic and amino acid synthesis via the pentose–phosphate pathway. Further significant reduction in the activity of α-GPDH in tamoxifen treated tumours is an indication that lipids are channelled into the pathways of carbohydrate metabolism rather than fat deposition.

As these changes in enzyme activities were observed at a time when the uptake of oestradiol was significantly reduced and that of 5α-DHT increased, a simple interpretation would be that oestradiol regulates PFK and G6PDH activities through an inhibitory control mechanism whereas it has a stimulatory effect on the activity of α-GPDH. However, it is contrary to the findings reported for DMBA-induced rat mammary tumours where oophorectomy reduced G6PDH activity [13] or for human breast tumours in primary cultures [27] in which addition of oestradiol-17β increased the enzyme activity in oestrogen dependent tumours. Further evidence that the changes in PFK, G6PDH and α-GPDH activities may not be mediated via the blocking of oestrogen receptors was obtained in another study of breast tumours from untreated Japanese patients, in which the activities of all the enzymes were similar to those observed for British patients treated with tamoxifen (Deshpande unpublished observation) and since the receptor levels are similar in Japanese and western women [28], it appears that the differences observed in treated and untreated tumours in British women may not be due to inhibition of oestrogenic stimuli. Therefore, it is possible that the drug acts directly on tumour cells or indirectly by altering the endocrine environment. Attempts are now being made to investigate these hypotheses.

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Surface Characteristics of Lymphoma Cells in Relation to Treatment, Response and Survival

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Abstract—*Tumour cell suspensions from 34 patients with non-Hodgkin's lymphoma were typed by E-rosetting and surface immunoglobulin techniques and the proportions of T and B cells so revealed were compared with the histological classification. Eighteen patients had a preponderance of B-cells and seven had a preponderance of T-cells; in three cases most cells had no marker and in six cases there were approximately equal mixtures of T- and B-cells The patients were treated and followed for periods of one to three years since analysis. The response to treatment and survival times have been correlated with the T and B properties. Patients with B-cell neoplasms appear to have a more favourable prognosis than those with T-cell neoplasms.*

INTRODUCTION

RECENT work on the properties of the lymphocyte cell membrane and the application of techniques to characterise and analyse the cells found in malignant lymphoproliferative disorders have provided a possible basis for a classification of those disorders as T, B or no-marker (null) cells [1–8].

It is not known yet whether this kind of classification has any practical value; in particular whether there is any difference between "T" and "B" cell tumours regarding the response to the treatment and survival; but recently [9] it has been shown that there is a poor prognosis in acute lymphoblastic leukaemia (ALL) with cells having T characteristics as opposed to the commoner form of ALL with null characteristics.

We have investigated 34 patients with lymphoid neoplasms using T- and B-cell markers and have looked at the response to treatment and the survival time of each group of patients as classified according to the T- and B-cell markers.

MATERIAL AND METHODS

Material

Thirty-four malignant lymphomas were studied between 1972 and 1975. They were classified in general according to Rappaport's classification [10]; eleven were nodular, one nodular and diffuse, the remainder were diffuse; seventeen were lymphocytic, eleven histiocytic, two mixed histiocytic/lymphocytic, two undifferentiated and one "immunoblastic sarcoma" (originally histiocytic diffuse [10]) and one immunoblastic lymphadenopathy progressing to mixed lymphocytic–histiocytic diffuse lymphoma.

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Methods

Fresh biopsy material from lymph nodes was used in all cases except for one where malignant cells from a pleural effusion were studied.

The lymph nodes were pressed through a steel mesh and the cells were made into suspensions in medium 199.

The cells were then washed three times in 199, counted and made up to a concentration of 4 $\times 10^6$/ml in medium 199 before the following techniques were performed.

Techniques

(a) Spontaneous E-rosette formation with sheep red blood cells (SRFC) was used as a T-cell marker.

(b) Fluorescent polyvalent antiserum to human immunoglobulin was used as a B-cell marker.

Both techniques were performed as previously described [11].

It should be mentioned that in reading the tests no distinction could be made between presumed neoplastic and normal cells in well-differentiated lymphoma but in poorly-differentiated lymphomas it was often possible to see that the rosetted or fluorescent cells had the morphology of the neoplastic cells.

Treatment

Cells from 17 patients were tested at initial diagnosis and before any form of treatment; 17 were tested at the time of a relapse. Four patients had only radiotherapy and 17 had only chemotherapy. Eleven had both chemotherapy and radiotherapy, while two patients with lymphoma of good prognosis were not treated during the period of this study.

Radiotherapy (mainly Cobalt) was given either locally to an upper mantle field or as total nodal irradiation. Chemotherapy consisted mainly of combinations of cytotoxic drugs. The majority of the patients had COPP (cyclophosphamide–vincristine–procarbazine–prednisolone) or COP (without procarbazine) or MOPP (mustine in place of cyclophosphamide) or MVPP (vinblastine in place of vincristine). Several patients had the same chemotherapy schedule on relapse or different ones including drugs such as methotrexate, bleomycin or adriamycin. Chlorambucil was also given either in combination with other drugs or as a single agent.

It will be appreciated that although rather diverse treatments were given, this is not an unusual circumstance and in no instance was the treatment influenced by the results of these studies.

RESULTS

The patients have been placed in 4 different groups according to the results of the T and B markers.

These groups were the following:

(a) Eighteen lymphomas in which the majority of the cells have detectable surface immunoglobulin. The proportion is between 60 and 88%, most of the remainder being accounted for as T-cells (see Table 1).

(b) Seven lymphomas in which 60–79% of the cells formed E-rosettes with a minority of surface-immunoglobulin positive B-cells (Table 2).

(c) In this group there were three patients in which the majority of the cells were negative to both tests (Table 3).

(d) Fourthly there were 6 patients with no majority of either T- *or* B-cells (Table 4).

In Table 5 the response to the treatment is presented with the number of patients from each group who achieved a complete remission, partial remission or showed no response; and in Figs. 1 and 2 the survival times are presented to

Table. 1. Lymphomas with B-cell predominance

No.	Name	Age	Histology	Tissue invest.	% SRFC	% Ig	Survival in months
1.	H.J.	25	PDLD	LN	11	88	60
2.	S.M.	45	PDLD	LN	11	61	21
3.	B.J.	72	PDLD	LN	3	80	17
4.	S.A.	76	WDLD	LN	15	72	26+
5.	P.W.	60	WDLN+D	LN	23	61	30
6.	C.T.	35	PDLN	LN	26	65	36+
7.	H.F.	60	PDLN	LN	25	66	27+
8.	R.C.	46	PDLN	LN	26	74	27+
9.	P.E.	68	PDLN	LN	25	75	$3\frac{1}{2}$
10.	M.W.	64	PDLN	LN	21	70	54+
11.	S.E.	41	PDLN	LN	30	61	137+
12.	M.S.	32	PDLN	LN	28	68	48+
13.	M.E.	48	PDLN	LN	28	63	37
14.	M.G.	64	MDLN	LN	25	75	$3\frac{1}{2}$
15.	B.P.	66	"Histiocytic" diffuse	LN	13	83	96
16.	S.J.	82	"Histiocytic" diffuse	LN	29	65	$4\frac{1}{2}$
17.	P.P.	40	"Histiocytic" diffuse	LN	28	65	42
18.	P.D.	24	"Histiocytic" diffuse	LN	22	60	36+

Table 2. Lymphomas with T-cells in predominance

No.	Name	Age	Histology	Tissue invest.	% SRFC	% Ig	Survival in months
1.	A.K.	42	"Histiocytic" diffuse	LN	60	25	3
2.	B.R.	65	"Histiocytic" diffuse	LN	68	31	5
3.	G.N.	54	"Histiocytic" diffuse	LN	77	9	8
4.	N.P.	25	"Histiocytic" diffuse	LN	66	12	20
5.	W.D.	40	Undifferentiated	Pleural effusion cells	62	20	11
6.	W.B.	68	Immunoblastic lymphadenopathy with progression	LN	70	27	$8\frac{1}{2}$
7.	W.R.	36	Immunoblastic sarcoma	LN	62	27	11

Table 3. Lymphomas with null-cells in predominance

No.	Name	Age	Histology	Tissue invest.	% SRFC	% Ig	Survival in months
1.	H.J.	42	PDLD	LN	16	1	4
2.	K.G.	55	PDLD	LN	15	12	6+
3.	C.A.	60	"Histiocytic" diffuse	LN	7	10	9

Table 4. Lymphomas with T and B cells in roughly equal numbers

No.	Name	Age	Histology	Tissue invest.	% SRFC	% Ig	Survival in months
1.	C.M.	72	PDLN	LN	46	48	22+
2.	W.F.	55	"Histiocytic" diffuse	LN	46	45	60+
3.	K.R.	49	"Histiocytic" nodular	LN	44	38	84
4.	S.R.	63	Undifferentiated	LN	39	26	43
5.	S.B.	38	Mixed H/L diff.	LN	37	39	18+
6.	S.B.	48	Mixed H/L nod.	LN	43	40	102+

Key to Tables 1–4
PDLD Poorly differentiated lymphocytic, diffuse.
PDLN Moderately differentiated lymphocytic, diffuse.
WDLD Well differentiated lymphocytic, diffuse.

Table 5. Response to treatment

Type of lymphoma	Number of cases			
	Complete remission	Partial remission	No response	Total
Lymphomas with B-cell characteristics	13	5	—	18
Lymphomas with T-cell characteristics	1	6	—	7
Lymphomas with negative T and B cell characteristics (null)	—	3	—	3
Lymphomas with roughly equal T and B cells	3	3	—	6

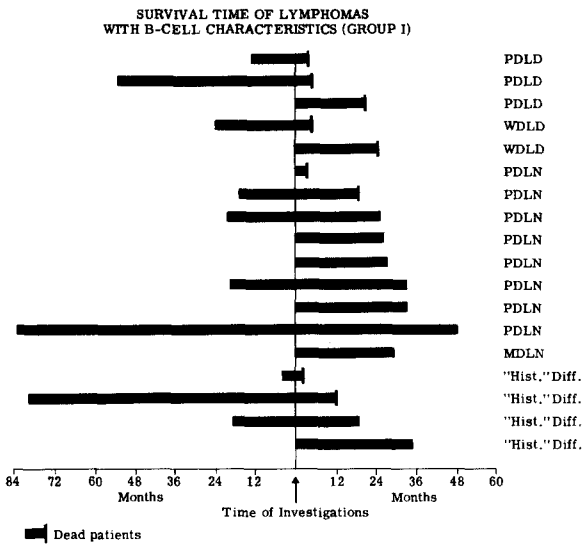

Fig. 1. Survival of patients before and after the time of investigation, where the majority of the lymph-node cells carried surface-immunoglobulin (B-cells).

dominant Hodgkin's disease where the great majority of the cells present are normal non-malignant reactive lymphocytes. The same may be true in some non-Hodgkin lymphomas especially perhaps those of mixed cellular type. On the other hand apparent heterogeneity of cell morphology may be associated with a single clone with otherwise uniform properties. Classification by morphology has also been misleading where tumours designated on account of cell size, abundance of cytoplasm etc. as "histiocytic" [10] have been shown by modern immunological techniques to have some properties of a lymphocyte.

Provided the data are not over-interpreted as indicating either that the cells in the majority are necessarily the neoplastic cells or that a distinctive B or T property necessarily indicates the cell of origin of the neoplasm, it is worth while examining lymphomas to see if relatively

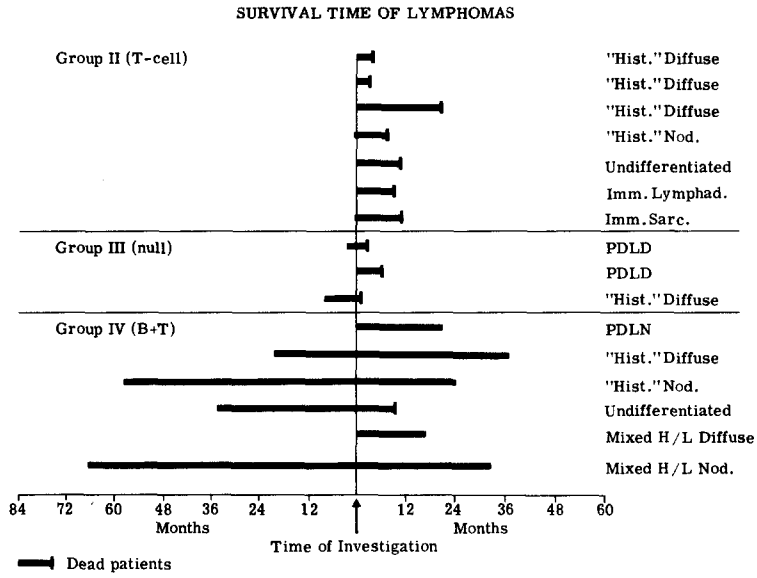

Fig. 2. Survival of patients before and after the time of investigation, where the lymphoma cells had T-cell characters, were "null", or where there was a mixture of cells.

illustrate the relations between histological type, surface properties and survival both from first diagnosis and from the time of testing.

DISCUSSION

The classification of lymphoid neoplasms is notoriously difficult and dependent on subjective criteria. The lymphomas comprise a wide spectrum of neoplasms of different degrees of malignancy and with diversities related to site, structure, cell-type and the age of the patient. Furthermore in any one neoplasm it may be difficult or impossible to distinguish the neoplastic cells from the interspersed non-malignant cells. An extreme example is lymphocyte pre-

simple tests of the cell-membrane can give useful information. In this study it was possible to analyse the patients into four main groups according to the type of cells in the tissue sampled which seemed to be in a majority. It is worth noting that in groups I, II and IV the proportion of cells with one of the two markers comprises 65–100 of the total and thus there was no evidence of the presence of double reacting cells. Nor is there any evidence to suggest that with the passage of time the proportion of cells with surface markers declines [6].

The main conclusion from our data is that patients with lymphomas having B-characteristics (which for convenience may be called B-cell lymphomas) have fared better than

those with T-cell lymphomas (Tables 1 and 2, Figs. 1 and 2); for example, all with T-cell lymphomas are dead, mostly within 1 yr, whereas the median survival for the B-cell cases will be in excess of two years. This difference is largely accounted for by the predominance in the B-cell group of tumours of "lymphocytic" type the majority being described as poorly differentiated. By contrast all the T-cell cases but one were of "histiocytic" or "immunoblastic" type. However even within the "histiocytic" category the prognosis for the B-cell patients appears to be rather better than for the T-cell patients. All 3 patients with cells of non-reactive "null" type did poorly, but it should be noted that this is not universally the case since the cells of typical childhood acute lymphoblastic leukaemia are also "null" and, with modern treatment, have a good prognosis. We have not included such cases in this report and the three "null" cases were in non-leukaemic adults.

The 6 patients with roughly equal mixtures of T and B cells have on the whole done well. They include the only two who were histologically classified as mixed histiocytic-lymphocytic (one nodular, one diffuse) and it is tempting to think that these are B-cell neoplasms with an infiltrate of non-neoplastic T-cells.

No therapeutic implications can be drawn from these findings since treatments were diverse and no comparisons are possible. However the fact that T-cell properties indicate a relatively poor prognosis suggests that all future therapeutic claims must be based on series of lymphomas analysed for T and B properties.

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Cellular Sensitization in Tumor-Bearing Mice to Soluble Extracts from Normal and Malignant Tissues

I. Common and Individually Distinct Antigens as Detected by Electrophoretic Mobility (EM) Test

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Abstract—*Mice bearing transplanted syngeneic tumors of different etiology are sensitized to at least 3 different types of antigen. One of them is related to the mouse encephalitogenic protein, the other ones are present in fractions which have been separated by Sephadex G-200 filtration following* 3 M KCl *technique. Sephadex G-200 filtration separated a common tumor antigen from individually distinct antigens. With regard to their* in vitro *specificity the latter ones behave like the distinct TATAs of chemically or physically induced tumors. The possible relationship between a fetal antigen and the cross-reacting tumor-antigen is discussed. Antigenicity was measured by the EM technique using lymph node cells from tumor-bearing animals.*

INTRODUCTION

IN A RECENT paper cross-reactions were shown among mouse tumors of different etiology by means of macrophage electrophoretic mobility (MEM) test [1]. The conclusion was drawn that mice bearing transplanted syngeneic tumors are sensitized to a common antigen which is detectable by the MEM-test and which is apparently different from the individually distinct tumor-associated transplantation antigens (TATA) of physically or chemically induced tumors.

Since the lymphocytes of tumor-bearing mice reacted with an encephalitogenic protein one of the cross-reacting antigens is presumably related to this protein. This resembles the situation in human tumor patients where the lymphocytes are sensitized to the human encephalitogenic protein [3]. Furthermore fetal antigens may be involved in the sensitization to tumors [2].

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In order to study the antigenic components which lead to sensitization in tumor-bearing animals different soluble preparations from normal and malignant tissues were tested by the EM technique.

MATERIAL AND METHODS

1. *Antigens*

Soluble tumor-associated antigens (TAA) were prepared according to Meltzer *et al.* [4] with some modifications [5]. In brief, tumor tissue from solid tumors was homogenized in 3 M KCl/PBS (tissue : buffer = 1 : 10 w/v) at pH 7.3. After 16 hr/4°C of extraction the suspension was centrifuged for 1 hr/100,000 ***g***. The clean supernatant was dialyzed against PBS (24 hr) followed by concentration using Visking tubes and polyethyleneglycol (type 20,000, Serva, GFR). This material was termed extract A. After centrifugation at 1 hr/200,000 ***g*** part of extract A was precipitated with an equal volume of saturated ammonium sulphate solution and

stored at 4°C/12 hr. The precipitate was collected by centrifugation 0.5 hr/30,000 *g*, dissolved in PBS, dialyzed against PBS (24 hr) and centrifuged if not clean. This part represents extract B. Part of the dissolved precipitate B was fractionated on Sephadex G-200 and 3 fractions C1, C2, C3 were collected (see Fig. 1). The protein solutions were concentrated by diafiltration to a content of 1–1.5% protein and stored at −25°C before using.

Mouse encephalitogenic protein (MEP) was prepared from mouse brain using the same technique previously described for rabbit encephalitogenic protein (REP) [1].

Fetal antigen was prepared from mouse embryos at an age between 12 and 18 days of gestation according to soluble tumor extract B.

2. *Donors of lymph node cells*

Donors of lymph node cells were inbred mice of the CBA/Bln and XVII/Bln strains. Tumor-bearing mice had i.m. transplanted tumors of different etiology. The size of the tumors varied between 10–20 mm dia. Tumors in XVII/Bln mice were ultraviolet-induced sarcomas UV-Bln/14306, UV-Bln/15264 and a GRAFFI-virus-induced leukemia LEU-Bln/Gr 101. Tumors in CBA/Bln mice were chemically induced by benzpyrene (sarcoma BP-Ros/01), methylcholanthrene (sarcoma MC-Bln/7149), nitrosomethyl-urea (leukemia NMU-Bln/131 and sarcoma NMU-Ros/01) and by implantation of polyvinyl chloride platelets (sarcoma PVC-Bln/11677). Furthermore a transplantable Mamma Ca in CBA mice (Ma-Bln/21224) was tested. The tumors are maintained by serial in transplantation in syngeneic recipients.

3. *Electrophoretic-mobility-(EM) test*

Lymphocyte sensitization was measured by EM-technique as previously described [6, 7], using guinea pig macrophages or tanned sheep red blood cells as indicator cells. Briefly, 1.3 $\times 10^6$ lymph node cells taken from tumor-bearing mice or controls were incubated with 200 μg antigen in 2 ml Eagle MEM for 1.5 hr/37°C. Supernatants from these mixtures were incubated with 1 ml of indicator cell suspension (10×10^6 tanned red blood cells or 7×10^6 macrophages) for 1.5 hr/23°C. Thereafter, the electrophoretic mobilities were measured in the cytopherometer (Fa. Opton, GFR). Results were expressed as slowing in percentage as compared with the electrophoretic mobility of the controls. As controls we used (1) indicator cells solely in the presence of antigen and (2) indicator cells after addition of supernatants from lymphocytes which had been incubated without antigen. A slowing $\geqq 5\%$ was arbitrarily considered positive. Data given in the tables are representing the mean values of repeated identical experiments.

RESULTS

Mice bearing transplanted syngeneic tumors are sensitized to an encephalitogenic protein which was isolated from mouse brain (Table 1). The reactions were also positive when extracts B from fetal mice were used as antigen. Normal mice did not react as well as were tumor bearers negative against B extracts from normal tissues.

When using A extracts from tumors, extensive cross-reactions were observed.

B extracts of tumors reacted preferably with lymphocytes from mice bearing the homologous tumor, i.e. extracts isolated from UV-Bln/14306 were positive in EM-test with lymphocytes from UV-Bln/14306 sarcoma-bearing mice and extracts from UV-Bln/15264 were positive with lymphocytes from UV-Bln/15264 tumor bearers. However, cross-reactions were frequently observed among both tumors (Table 2).

In comparative studies with extracts A, B and C2 from 3 tumors each a separation of antigenic activities could be observed (Table 3). According to the results in Table 2 the B extracts from tumors showed extensive cross-reactions. Thus, cross-reactions were observed among the sarcomas induced by benzpyrene, methylcholanthrene and nitrosomethyl-urea. However, individually distinct antigenicity appeared in fraction C2. Extract A reacted in a non-specific way even with lymphocytes from normal donors. In Fig. 1 the Sephadex G-200 elution profile of sarcoma MC-Bln/7149 (extract B) is correlated with the pattern of antigenic distribution in the fractions. Lymphocyte donors were mice bearing the homologous tumor MC-Bln/7149 and mice bearing tumor BP-Ros/01. Three peaks C1, C2, C3 according to mol. wt of >180,000, 120,000–180,000, 50,000–80,000, were obtained after Sephadex G-200 filtration of tumor extract B. Whereas C1 was antigenically inactive, C2 revealed individually distinct antigenicity in that it only reacted with lymphocytes from mice bearing the homologous tumors. C3 appeared to be the cross-reacting antigen. It showed positive reaction for instance also with lymphocytes from BP-Ros/01 tumor bearers.

In order to rule out non-specific activity of the A, B and C extracts they were tested against lymphocytes of normal animals (not shown in the table). Except extract A the slowing of

indicator cells was less than 3% which is in the range of the controls.

C2 and C3 fractions isolated from several other tumors were tested against lymphocytes of different tumor bearers (Table 4). The data show in conformity with those in Fig. 1 that C2 contains the individually distinct antigen of the tumors while C3 has a cross-reacting common tumor antigen.

DISCUSSION

Mice bearing transplanted tumors are obviously sensitized to a number of different antigens which are associated with the neoplasm. Sensitization to different kinds of antigen is preferentially demonstrable by *in vitro* techniques such as the EM-test. Our data show that at least 3 types of antigen are involved [1] an

Table 1. Lymphocyte sensitization of tumor bearing mice to mouse encephalitogenic protein (MEP) and mouse fetal antigen as measured by the EM-technique

Lymphocyte from donors bearing	MEP	Fetal antigen	Normal tissue antigens				
			Heart	Lien	Kidney	Liver	Lung
UV-Bln/14306*	+14.7 (4)	+14.8 (4)	−1.2 (2)	−0.5 (2)	−2.2 (2)	−1.9 (2)	+0.3 (2)
UV-Bln/15264*	+11.8 (16)	+11.1 (11)	0 (2)	+2.4 (2)	+1.3 (2)	−0.3 (2)	+1.7 (2)
LEU-Bln/Gr101*	+ 9.6 (16)	+11.7 (15)	−1.1 (2)	−3.3 (2)	−1.5 (2)	−3.0 (2)	+1.0 (2)
Ma-Bln/21224†	+10.3 (7)	+11.2 (8)	n.t.‡	+2.5 (2)	+1.9 (2)	+0.1 (2)	−1.0 (2)

(Values are given in percentage of slowing as compared to the controls. The numbers of repeated experiments are put in brackets).
*Lymphocytes tested 7–12 days after inoculation of tumor cells.
†Lymphocytes tested 13–18 days after inoculation of tumor cells.
‡n.t. = not tested.

Table 2. Lymphocyte sensitization of tumor bearing mice to A- and B-extracts of u.v.-light induced sarcomas

Lymphocytes from donors bearing	A-extracts		B-extracts	
	UV-Bln/14306	UV-Bln/15264	UV-Bln/14306	UV-Bln/15264
UV-Bln/14306	+12.6 (11)	+3.0 (13)	+13.8 (9)	+12.1 (9)
UV-Bln/15264	+7.8 (11)	+11.1 (15)	+11.2 (11)	+14.8 (12)

(Values are given in percentage of slowing as compared to the controls. The numbers of repeated experiments are put in brackets).

Table 3. Antigenic activity of extracts A, B and C_2 of chemically induced sarcomas as measured by EM-technique

Lymphocyte donors	TAA								
	BP-Ros/01			NMU-Ros/01			MC-Bln/7149		
	A	B	C2	A	B	C2	A	B	C2
Controls									
CBA/Bln mice	7	2	−1	5	1	1	6	2	0
BP-Ros/01									
CBA/Bln mice	13	20	19	9	11	4	7	9	2
NMU-Ros/01									
CBA/Bln mice	8	14	2	12	17	13	7	10	2
MC-Bln/7149									
CBA/Bln mice	9	10	3	5	10	3	10	19	16

Values are given in percentage of slowing as compared to the controls (rounded values).

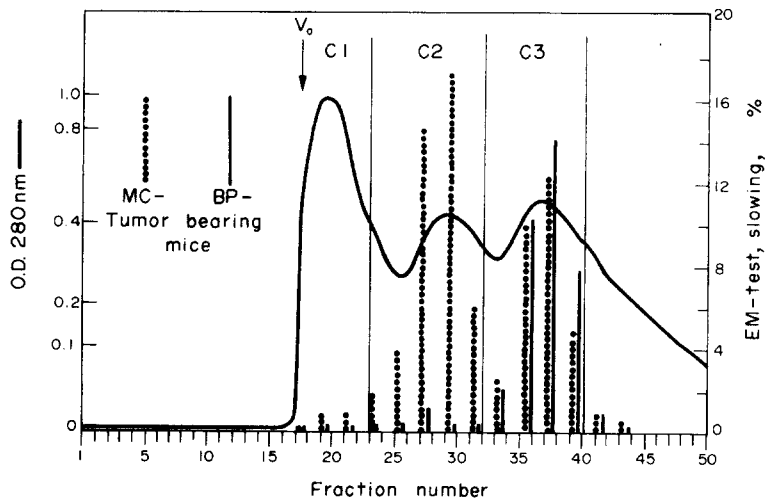

Fig. 1. Correlation of elution pattern and antigenic activity of the fractions as measured by EM technique. Extract B of sarcoma MC-Bln/7149 was fractionated on Sephadex G-200 and the fractions obtained were tested against lymphocytes from animals bearing the homologous tumor (MC-Bln/7149) or another tumor (BP-Ros/01).

Table 4. Antigenic activity of partially purified fractions C2 and C3 of tumor extracts as measured by EM-technique

Lymphocyte donor	TAA									
	BP-Ros/01		NMU-Ros/01		NMU-Bln/131		PVC-Bln/11677		MC-Bln/7149	
	C2	C3	C2	C3	C2	C3	C2	C3	C2	C3
Control CBA/Bln mice	0	−1	1	0	−1	0	0	1	0	−1
Control XVII/Bln mice	−1	−1	−1	−1	2	0	0	−1	−1	−1
BP-Ros/01 CBA/Bln mice	19	14	4	19	4	12	3	18	2	15
NMU-Ros/01 CBA/Bln mice	2	12	13	10	3	9	2	10	2	14
NMU-Bln/131 CBA/Bln mice	1	10	1	11	20	15	1	13	2	12
PVC-Bln/11677 CBA/Bln mice	2	15	2	13	1	10	19	14	0	16
MC-Bln/7149 CBA/Bln mice	3	13	1	12	2	16	2	12	16	19
UV-Bln/15264 XVII/Bln mice	−1	9	−1	16	0	10	−2	12	−2	12
LEU-Bln/Gr 101 XVII/Bln mice	1	17	0	18	−2	13	−2	14	0	17

Values are given in percentage of slowing as compared to the controls (rounded values).

antigen which is identical with or related to the encephalitogenic protein, [2] the cross-reacting antigenic component C3 of tumors and [3] fraction C2 which shows individually distinct antigenicity. Whether C3 corresponds with the cross-reacting fetal antigen is not yet known. The similar pattern of reactivity obtained with C3 and the fetal antigen is striking. Data published in a previous paper [1] indicated the sensitization of tumor-bearing mice to rabbit encephalitogenic protein but not to human encephalitogenic protein. Here is also shown the sensitization to mouse encephalitogenic protein. Furthermore, human tumor patients are sensitized to human encephalitogenic protein but not to rabbit encephalitogenic protein (unpublished results). Thus, encephalitogenic proteins of related species seem to have common antigenic components.

When using B extracts as antigen no clear-cut results were obtained with regard to the pattern of reaction. Specificity of reactions dominated, i.e. the reactivity of antigen in the homologous tumor-host system was generally higher in comparison with the reactivity against another tumor. This was particularly evident in the case of the UV tumors 14306 and 15264.

In an attempt to separate the presumed antigenic components the B extracts were filtered through a Sephadex G-200 column. Identical elution patterns were obtained with several B tumor extracts an example of which is shown in Fig. 1. In this way, cross-reacting and individual distinct antigenic extracts could be separated. It remains an open question whether the fraction containing the individually distinct antigenic component C2 is related to the individually distinct antigens of chemically or physically induced tumors. Because of the characteristic pattern of reaction C2 is now also being studied in cross-resistance tests *in vivo*. In our hands, B extracts from tumors are immunogenic in syngeneic recipients and produce tumor-specific transplantation resistance [8].

The antigenic activity of the fractions C2 and C3 corresponds well with the profile of 2 protein peaks. It should be mentioned, however, that the active fractions can be best selected according to the EM test results. There are a number of reports now on the fractionation of tumor extracts [9–12]. Hypertonic salt extraction and/or ammonium sulphate precipitation were the most frequently used techniques. However, the results obtained are not directly comparable since the antigenic activities have been tested by different immunological methods.

Our experiments show that the EM technique is suitable to the measurement of cellular sensitization as well as to the analysis of antigenic components in tissue extracts.

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Cross-Reactivities of Ethylnitrosourea-Induced Neurogenic Rat Tumors Studied by *in vitro* Assays For Cell-Mediated Immunity

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Abstract—*Neurogenic tumors were induced in inbred Wistar rats by the transplacental administration of ethylnitrosourea (ENU). The tumors were transplantable in syngeneic rats and they could easily be grown* in vitro.

Migration inhibition tests (MIT) were carried out with KCl extracts of several neurogenic tumors, a spontaneous mammary carcinoma and of various normal adult and fetal tissues. Thymocytes and lymph node cells for these tests were obtained from primary tumor hosts, recipients of transplanted tumors and from rats hyperimmunized with Mitomycin C treated tumor cells.

An indirect ^{51}Cr-release test as well as an indirect DNA inhibition test were carried out with culture supernatants derived from mixed cultures of neurogenic tumor cells and sensitized or non-sensitized lymphoid cells.

All three assays revealed tumor associated antigens with cross-reactions between various neurogenic tumors. Cross-reactions were also demonstrated by MIT between neurogenic tumors and fetal tissues. A peripheral neurinoma showed cross-reaction with adult brain and a trigeminal nerve neurinoma cross-reacted with the spontaneous mammary carcinoma. None of the neurogenic tumors cross-reacted with adult liver. Thus, ENU induced neurogenic tumors share one or several common antigens which, however, are not tumor specific.

The reactivity of lymphoid cells derived from tumor bearing rats was considerably smaller than that of lymphoid cells from hyperimmunized rats. It was also found that the reactivity of lymphoid cells from thymus and lymph nodes was considerably stronger than that of splenocytes.

INTRODUCTION

THE INDUCTION of nervous system tumors by methylnitrosourea in rats was first reported by Druckrey *et al.* [1] in 1964. Since then the oncogenic effect of many different nitroso derivatives has been described by a number of authors [2]. The oncogenic effect of ethylnitrosourea (ENU) administered by the transplacental route is of particular interest. Almost all rats exposed to certain amounts of ENU in the late phase of embryonic life develop tumors in the nervous system later in adult life.

The importance of the biochemical and immunological characterization of these tumors has been stressed [3]. Generally speaking, individual specificities are typical of tumor associated antigens in chemically induced tumors. However, conflicting views on this point are held in the case of ENU induced neurogenic tumors.

In the present study, the cross-reactivities of ENU induced neurogenic rat tumors were studied by various *in vitro* tests for cell mediated immunity.

MATERIAL AND METHODS

Inbred rats of the Wistar strain (Wistar/AfHanMolFib) were used throughout these experiments. The animals were bred and maintained under "minimal disease" conditions at the Fibiger Laboratory.

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Ethylnitrosourea (ENU) was obtained from Nakarai Chemicals, (Kyoto, Japan).

Induction and transplantation of tumors

Sixty milligrams per kilogram of ENU were injected intraperitoneally into pregnant rats in the third week of gestation. ENU was first dissolved in the ethanol and then diluted with 10 vol of phosphate buffered salt solution (PBS). The off-spring were observed until spontaneous death or until the preterminal stage.

In 99 rats a total of 110 tumors were demonstrated. In most cases the histological diagnosis was either malignant neurinoma (41%) or malignant oligodendroglioma (25%), but mixed gliomas. astrocytomas, ependymomas and meningiomas were also seen.

The transplantability of 9 neurogenic tumors and the mammary carcinoma was tested in newborn as well as adult recipients. They all produced tumors and permanent tumor lines were established in 2–3 months old Wistar rats of both sexes.

Explantation and cultivation of tumors

Small (1–2mm) pieces of tumor tissue were placed at the bottom of T-25 or T-75 plastic flasks and preincubated for 1–2 hr at 37°C and 5% CO_2. When the fluid medium after this preincubation was added carefully to the cultures, most of the explants would remain fixed to the bottom. The medium (Fib 41B) was a modification of Eagle's medium (MEM) with a two-fold concentration of the aminoacids, a four-fold concentration of vitamins and glutamine and with a 35% increase of the glucose concentration. This medium was fortified with 15% fetal calf serum (FCS). The cultures were incubated in a humid 5% CO_2 incubator for varying periods of time. For subcultivation, the cells were brought into suspension with the aid of trypsin. The medium was renewed 2–3 times weekly.

Tissue extracts

Tissue extracts were prepared with 3 M KCl [4, 5] from the following tissues: One malignant neurinoma from a peripheral nerve (T1) and two from the trigeminal nerve (T2 and T3), a mixed glioma (T4) and a spontaneous Wistar rat mammary carcinoma (T9). In addition extracts were prepared from adult brain and liver, 2 weeks fetal brain, 2 weeks fetal mixed tissues without brain and spinal cord, 1 week whole fetuses. The extracts were stored at −80°C and thawed immediately before use. No sample was subjected to more than one cycle of freezing and thawing.

Immunization procedures

Tumor cell suspensions were prepared by mild homogenization of small pieces of tumor tissue in a Daunce glass homogenizer followed by filtration through a monolayer of Nylon mesh (pore size 80 μm) and pretreated with 250 μg mitomycin C (MMC) per 2 ml PBS containing 10^7 cells. After incubation for 3 hr at 37°C the cells were resuspended in PBS and injected subcutaneously into 2–3 months old Wistar rats. Each rat received 10^7 MMC treated cells 5 times at weekly intervals. Seven to ten days after the last injection the rats were sacrificed and thymus and lymph nodes (axillar, brachial, inguinal, parathymic and submandibular) were removed. A suspension of lymphoid cells was prepared following the same procedure used for the tumor material.

Migration inhibition test (MIT)

Mixed thymus and lymph node cells (T + LN) were washed 5 times in PBS and once in Parker's medium 199 with 15% heat inactivated horse serum, 100 i.u./ml of penicillin and 100 μg/ml of streptomycin. This medium also served as migration medium. After the last centrifugation, the packed lymphoid cells were resuspended in 6 vol of the migration medium and sucked into 20 μl Drummond capillary tubes with a vacuum pump. The capillary tubes were presealed at the upper end by heating. When filled with lymphoid cell suspension, the capillary tubes were turned round and centrifuged at $750 \times g$ for 3 min. Finally they were cut at the cell-fluid interphase and fixed to the bottom of the migration wells of Sterilin No. 308 migration plates (Richmond, U.K.) with silicon grease.

Each well received 0.4 ml of migration medium. Antigen (ag) was added at a concentration of 100 μg protein/ml, which in pilot studies was found to be optimal. Protein determinations were made with Lowry's method. After sealing of the wells with coverslips, the migration plates were incubated for 22–24 hr at 37°C in a CO_2 incubator. After incubation the migration plates were placed in a Liesegang projector and the circumference of the projected migration zones were drawn on paper and cut out. The migration areas (MA) were quantitated by weighing, and the average results in each experimental group were calculated.

In each group, 6 migration cultures were studied. The migration inhibition index (MII) for each migration culture was calculated as:

$$\text{MII} = \frac{\text{MA with ag}}{\text{Av. MA without ag}}$$

A significant difference between the average MII with lymphoid cells from sensitized and non-sensitized rats was established by P values $\leqq 0.05$ by the Student's two tailed t-test.

Indirect ^{51}Cr release test

The immune response to ENU induced tumor cells was also studied by an indirect cytotoxicity test, using ^{51}Cr labeled C3H mouse ascites tumor cells—C3H-L1/a [6]—as test cells.

In the first step of this test 4×10^5 neurogenic tumor cells were seeded in 5 ml Fib 41B without FCS in T-75 tissue culture flasks and incubated overnight at 37°C in a CO_2 incubator. Sensitized and non-sensitized lymphoid cells were subsequently added at a lymphocyte/tumor cell ratio of 200/1. After 48 hr of additional incubation, the supernatant culture medium was harvested and concentrated 100 times in dialysis bags with Aquacide-III. The nutritional conditions were finally restored by redilution of the concentrate (to the desired concentration) with fresh Fib 41B with 5% FCS.

During the second step C3H-L1a test cells were suspended in Fib 41B with 5% FCS at a concentration of 5×10^6 cells per ml. The test cells were labeled with 175 μCi of ^{51}Cr (Amersham) for 2 hr at 37°C. After labeling, the test cells were washed 5 times with Fib 41B with 5% FCS. The first washing was carried out with ice cold medium.

Finally the test cells were resuspended in fresh medium at a concentration of 2×10^6 cells/ml. From each suspension a sample of 10 μl was withdrawn and placed directly in vials for counting in a Selektronik gamma counter (Hørsholm, Denmark) in order to determine total radioactivity.

Other 100 μl samples were mixed with 500 μl tissue culture supernatants prepared as described in sterile test tubes. Fresh medium was used for the demonstration of spontaneous ^{51}Cr-release. The mixtures were incubated for 48 hr at 37°C. After centrifugation at $750 \times g$ for 10 min, 500 μl samples of the supernatant fluid were withdrawn and placed in the gamma counter. The relative ^{51}Cr-release from cells treated with the test supernatants was calculated as percentage of the total activity (T.A.) after correction for spontaneous release (S.R.):

$$\frac{\bar{x} \text{ counts/min [test]} - \bar{x} \text{ counts/min [S.R.]} \times 6}{\bar{x} \text{ counts/min [TA]} - \bar{x} \text{ counts/min [S.R.]} \times 5} \times 100$$

Indirect DNA inhibition test

This test will be described in greater detail elsewhere. It is carried out in two steps. In the first step lymphoid cells react with cultured neurogenic tumor cells using the same method as in the indirect ^{51}Cr-release test except that the incubation time is reduced to 16–18 hr.

In the second step microcultures of hamster PM_1 cells [7] are prepared in Linbro Disposo Trays (FB-48 clear type) obtained from Dansk Mikrobiologisk A/S, Århus, Denmark. Each well is seeded with 2×10^4 tumor cells in 0.2 ml Fib 41B with 10% FCS. The cultures are preincubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air until a uniform monolayer of cells covers the bottoms of the wells. This usually takes 12–24 hr. After washing 3 times with Fib 41B each well receives 0.2 ml of the supernatant media prepared as described during the first step of the test.

The microcultures are replaced in the CO_2 incubator and after an additional period of incubation of 17 hr the cultures are labeled for 3 hr with (6-^{3}H) thymidine (TRA 61—Radiochemical Centre, Amersham. England) which is added to yield a final concentration of 0.2 μM and a specific activity of 40 μCi/ml.

At the end of the labeling period, 25 μl of a 22 mM cold thymidine solution in Fib 41B are added. After a final period of 30 min incubation, the medium is removed and the cultures washed twice with PBS before fixation of the cells for 5 min in ethanol/glacial acetic acid (4:1). After fixation the microcultures are treated with TCA (5%) for 5 min and washed with fixative before air-drying at room temperature for 20–30 min.

Finally the bottoms of the wells with all the test cells are cut off and placed in vials containing 10 ml of toluene with PPO (4 g/l) for counting in a Beckman LS-100 scintillation counter. Each vial is counted twice for 10 min.

RESULTS

Migration inhibition test (MIT)

MIT was carried out as described with KCl extracts of the following tumor material: T1, T2, T3, T4, a mixture of T1 with a few other neurinomas and a spinal ependynoma (Tmix), and a spontaneous rat mammary carcinoma. Furthermore, extracts were prepared from various normal adult and fetal tissues as previously mentioned. Lymphoid cells were obtained from (1) primary tumor hosts, (2) recipients of transplanted tumors and (3) from rats

hyperimmunized with MMC treated tumor cells.

Lymphoid cells derived from 39 primary hosts carrying various types of neurogenic tumors were tested against 3 M KCl extracts of transplanted neurogenic tumors (T1, T2, T3, Tmix) and of normal adult brain. In 19 cases a significant inhibition (MA with ag < MA without ag) was obtained with at least one of the extracts. All extracts including extracts of normal brain were active. The percentage of positive tests varied from 36 (T3) to 60% (brain) as shown in Table 1.

*Table 1. Migration inhibition test with lymphoid cells from the original tumor bearing rats**

Source of antigen	T-mix	T1	T2	T3	Brain
No. of pos. cases/total	4/7	8/20	4/7	5/14	3/5
Percentage	57	40	57	36	60

*19/39 rats showed positive migration inhibition against at least one of the tumors.

The results of these pilot studies might be explained by the presence of a common antigen shared by a number of ENU induced tumors and normal brain. However, they do not allow any conclusions concerning the specificity of the reactions.

Table 2 shows the results of some experiments in which the sensitized lymphoid cells were derived from rats transplanted s.c. with T1 (1.—2. pass), T2 (1.—6. pass) and T3 (1.—3. pass). Most of the donors were moribund, carrying huge tumors. The specificity of the reactions was tested by comparison to T + LN lymphoid cells from normal, non-sensitized adult rats. 3 M KCl extracts of transplanted T1, T2 and T3 tumors served as antigen.

As a whole, migration inhibition was modest. MII values which represent a significant difference between MI ± ag are underlined in the Table.

In a number of cases a significant difference was established between lymphoid cells from tumor-bearing and normal rats, indicating a specific reaction.

A significant inhibition of MA by tumor antigen combined with a significant difference between MII values obtained with sensitized and nonsensitized lymphoid cells was seen in the following combinations. Anti-T1 lymphocytes challenged with T1 and T3 extracts. Anti-T2 lymphocytes challenged with T1 extracts. Anti-T3 lymphocytes challenged with T1, T2 and T3 extracts.

These results indicate a cross reaction between T1 and T3.

For the further elucidation of these antigenicities, lymphoid cells were prepared from rats immunized 5 times with MMC treated tumor cells. KCl extracts of malignant and nonmalignant tissues served as antigen. The results are presented in Tables 3–5. Most of the values are based on 6 migration cultures.

A majority of extracts which served as antigen in these experiments caused a significant inhibition of the migration of anti-T1, anti-T2 as well as anti-T3 lymphoid cells (MA + ag < MA − ag).

The specificity of the reactions is demonstrated by a significant difference between the MII values obtained with sensitized and nonsensitized lymphoid cells as shown in the Tables. However, it also appears from the tables that the sensitized lymphoid cells reacted not only with malignant extracts, but in some cases also with fetal extract and in a single case with extract from adult brain.

The cross reactions observed in these experiments are summarized in Table 6 from which it is seen that T1 and T3 seem to share at least one common antigen which also is present in T4 and mixed fetal tissues of 1 week of gestation. Extracts of a spontaneous mammary carcinoma (T9) and fetal brain (2 weeks of gestation) caused a specific inhibition of anti-T3 lymphoid

Table 2. Migration inhibition test with lymphoid cells from rats carrying transplanted neurogenic tumors

Antigen	T1		T2		T3	
Lymphoid cells	anti-T1	control	anti-T1	control	anti-T1	control
MII	0.92 ± 0.06*	1.06 ± 0.08	0.98 ± 0.07	1.16 ± 0.11	0.81 ± 0.06	0.92 ± 0.08
P-value	0.01–0.005		0.025–0.0125		0.05–0.025	
Lymphoid cells	anti-T2	control	anti-T2	control	anti-T2	control
MII	0.90 ± 0.07	1.05 ± 0.06	0.89 ± 0.13	1.13 ± 0.16	0.79 ± 0.15	0.83 ± 0.11
P-value	0.05–0.025		0.10–0.05		0.40–0.35	
Lymphoid cells	anti-T3	control	anti-T3	control	anti-T3	control
MII	0.92 ± 0.10	1.01 ± 0.05	0.84 ± 0.19	0.99 ± 0.19	0.71 ± 0.12	0.87 ± 0.13
P-value	0.05–0.025		0.15–0.10		0.0125–0.01	

*Underlined values represent statistically significant inhibition of MA with ag as compared to MA without ag.

Table 3. Migration inhibition test with T1-sensitized lymphoid cells

Antigen	MII		
	Sensitized lymph.	Control lymph.	*P*-value
T1	0.81 ± 0.07*	0.99 ± 0.09	< 0.0005
T2	0.88 ± 0.12	1.01 ± 0.03	0.0125–0.01
T3	0.75 ± 0.08	0.87 ± 0.11	0.0025–0.0005
T4	0.85 ± 0.08	0.97 ± 0.04	0.025–0.0125
Spontaneous mammary cancer (T9)	0.69 ± 0.17	0.85 ± 0.08	0.10–0.05
2 weeks fetus	0.71 ± 0.33	0.96 ± 0.04	0.15–0.10
2 weeks f.brain	0.87 ± 0.13	1.02 ± 0.11	0.10–0.05
1 week fetus	0.69 ± 0.10	0.90 ± 0.06	0.025–0.0125
Adult brain	0.82 ± 0.12	0.95 ± 0.09	0.025–0.0125
Adult liver	0.84 ± 0.20	0.91 ± 0.18	0.30–0.25

*Underlined values represent statistically significant inhibition of MA with ag as compared to MA without ag.

Table 4. Migration inhibition test with T2-sensitized lymphoid cells

Antigen	MII		
	Sensitized lymph.	Control lymph.	*P*-value
T1	0.80 ± 0.08*	0.96 ± 0.05	0.025–0.0125
T2	0.77 ± 0.15	0.92 ± 0.14	0.10–0.05
T3	0.77 ± 0.13	0.96 ± 0.24	0.10–0.05
T4	0.88 ± 0.14	1.07 ± 0.16	0.10–0.05
Spontaneous mammary cancer (T9)	0.67 ± 0.20	0.83 ± 0.11	0.15–0.10
2 weeks fetus	0.74 ± 0.19	0.86 ± 0.16	0.25–0.20
2 weeks f.brain	0.87 ± 0.03	1.10 ± 0.01	0.005–0.0025
1 week fetus	0.67 ± 0.17	0.81 ± 0.17	0.20–0.15
Adult brain	0.75 ± 0.11	0.92 ± 0.20	0.25–0.20
Adult liver	0.64 ± 0.19	0.84 ± 0.13	0.15–0.10

*See Table 3.

Table 5. Migration inhibition test with T3-sensitized lymphoid cells

Antigen	MII		
	Sensitized lymph.	Control lymph.	*P*-value
T1	0.88 ± 0.10*	0.98 ± 0.05	0.025–0.01
T2	0.87 ± 0.14	0.97 ± 0.14	0.15–0.10
T3	0.78 ± 0.11	0.93 ± 0.11	0.01–0.005
T4	0.86 ± 0.11	1.01 ± 0.08	0.025–0.0125
Spontaneous mammary cancer (T9)	0.61 ± 0.12	0.83 ± 0.10	0.01–0.005
2 weeks fetus	0.77 ± 0.10	0.91 ± 0.04	0.025–0.01
2 weeks f.brain	0.84 ± 0.05	1.06 ± 0.07	0.01–0.005
1 week fetus	0.65 ± 0.0005	0.91 ± 0.08	0.05–0.025
Adlt brain	0.70 ± 0.18	0.77 ± 0.20	0.30–0.20
Adult liver	0.77 ± 0.18	0.88 + 0.13	0.20–0.15

*See Table 3.

Table 6. Cross-reactions of sensitized lymphoid cells with extracts from various ENU induced neurinomas (T1, T2, T3) and mixed glioma (T4), from spontaneous mammary carcinoma (T9) and from various fetal and adult rat tissues

Extract	T1	T2	T3	T4	T9	1 week fetus	2 weeks fetus*	2 weeks f.brain	Adult brain	Adult liver
Lymphoid cells sensitized to										
T1	+	+	+	+	−	+	−	−	+	−
T2	+	−	−	−	−	−	−	+	−	−
T3	+	−	+	+	+	+	+	+	−	−

*Without brain and spinal cord.

cells while the difference between non-sensitized and anti-T1 lymphoid cells was only on the borderline of statistical significance.

Extracts of T1 and fetal brain tissue cross-reacted with anti-T2 lymphoid cells, while T2 extract only reacted with anti-T1 lymphoid cells. The antigenic strength of this tumor seems rather weak.

Indirect ^{51}Cr-release test

The cytotoxicity of the supernatant medium from mixed cultures of T1 or T3 tumor cells propagated *in vitro* and sensitized or non-sensitized lymphoid cells was tested by the indirect ^{51}Cr-release test as described. From Table 7 it appears that T1 tumor cells stimulated T2 and T3 sensitized T + LN lymphoid cells but not T1 sensitized lymphocytes to produce a cytotoxic supernatant, while sensitized spleen cells were without any effect.

T3 tumor cells stimulated T + LN lymphoid cells sensitized to T1 and T3 in a similar way. Sensitized spleen cells were less efficient.

Even though no reaction between T1 and anti-T1 could be demonstrated in this experiment, the cross-reactions demonstrated by MIT were confirmed.

Indirect DNA inhibition test

The production of DNA inhibiting factors by T1, T2 and T3 sensitized lymphoid cells mixed with T1 or T3 tumor cells propagated *in vitro* was tested as described. The specific DNA inhibition (SDI) was calculated as per cent inhibition in comparison to control supernatants derived from mixed cultures of tumor and non-sensitized lymphoid cells.

As shown in Table 8, media from mixed cultures of all 3 types of sensitized lymphoid

Table 7. Indirect ^{51}Cr-release test

Target cells	Lymphoid cells	^{51}Cr-release cp 100 sec.	*P*-value	% release after deduction of spont. release
T1 (CR26-1)	anti-T1 (T + LN)*	382532 ± 20260	NS‡	22.52
	anti-T2	404748 ± 20150	0.025–0.0125	30.44
	anti-T3	452299 ± 21541	< 0.0005	47.39
	control	380153 ± 6545		21.67
	anti-T1 (Spl)†	411302 ± 15825	NS	32.77
	anti-T2	395613 ± 32539	NS	27.18
	anti-T3	430974 ± 68196	NS	39.79
	control	415118 ± 10043		34.13
T3 (CR28-1)	anti-T1 (T + LN)	341729 ± 15665	< 0.0005	7.98
	anti-T2	357200 ± 12171	< 0.0005	13.49
	anti-T3	428582 ± 13983	< 0.0005	38.93
	control	289512 ± 12874		−10.64
	anti-T1 (Spl)	310280 ± 8741	NS	−3.23
	anti-T2	351893 ± 16051	0.025–0.0125	11.60
	anti-T3	437154 ± 12866	< 0.0005	42.00
	control	321364 ± 22064		0.72

*T + LN = thymic cells + lymph node cells.
†Spl = spleen cells.
‡NS = non-significant.

Table 8. Indirect DNA inhibition test

Target	Lymphoid cells	Counts/min	*P*-value	SDI*
T1 (CR26-1)	anti-T1 (T+LN)	19750±2163	0.01–0.005	19%
	anti-T2	18578±2989	0.005–0.0025	25%
	anti-T3	17776±2489	0.0025–0.0005	27%
	control	24254±2766		
T3 (CR28-1)	anti-T1 (T+LN)	21313±3382	0.025–0.0125	15%
	anti-T2	25869±4383	0.35–0.30	−3%
	anti-T3	17056±3639	<0.0005	32%
	control	25068±2153		

*Specific DNA inhibiting effect calculated as per cent inhibition in comparison to control.

cells and T1 tumor cells had a significant DNA inhibiting effect with SDI values varying from 19 to 27%.

Similar results were obtained with T3 tumor cells and anti-T1 or anti-T3 lymphoid cells (SDI: 15 and 32%, respectively), but not with anti-T2 lymphocytes.

DISCUSSION

The immunogenicity of ENU induced neurogenic tumors has been tested in transplantation experiments which have shown that young recipients are more susceptible than older ones [8, 9]. Furthermore, intracerebral transplantations have been reported to yield a higher rate of takes than subcutaneous inoculations [8]. These observations indicate the presence of transplantation antigens on the ENU induced tumor cells.

In the present investigation the antigenicity of ENU induced neurogenic rat tumors was confirmed by direct migration inhibition tests as well as by indirect ^{51}Cr-release and DNA inhibition tests. Cross-reactions were observed between peripheral nerve neurinoma (T1), trigeminal nerve neurinoma (T2, T3) and mixed glioma (T4).

The immunogenic effect of T2 was quite low, but lymphocytes sensitized to this tumor did respond to extracts of the T1 tumor in the migration inhibition test and to T1 cells in the indirect ^{51}Cr-release and DNA inhibition tests. Cross-reaction with T3 tumor cells was seen in the ^{51}Cr-release test. The cross-reactions between T1 and T3 were confirmed by all 3 types of assays. In MIT these tumors also cross-reacted with T4.

These results indicate that the ENU neurogenic tumors share at least one common antigen of varying strength. Similar results have been published by Cornain *et al.* [10], who used a microcytotoxicity assay. However, by selective elimination of spleen cells carrying surface Ig and C3 receptor, a T-cell enriched lymphocyte population was obtained which in the microcytotoxicity assay revealed distinct antigenic specificities, confirming the results of *in vivo* rejection tests.

In our investigation thymocytes and lymphonode cells were used. For unknown reasons spleen cells were found to be less responsive. Whether a purification of the mixed T+LN population will disclose tumor distinct specificities remains to be shown. The direct MIT will probably be suitable for such a study. However, in a few preliminary studies, we have been unable to demonstrate such specificities *in vivo* by active protection and tumor neutralization tests.

Toh [11] failed to show cross-reactions between Schwannomas and gliomas by a direct mixed culture method, which was taken as a sign of the immunogenic weakness of the Schwannoma tumor cells. It is possible that the method employed was not sensitive enough to detect the antigens of the Schwannoma cells, but the negative results might also be due to the fact that the lymphoid cells were derived from tumor bearing rats in which circulating antigens or immune complexes could have caused antigenic inhibition of the effector cell.

In the present investigation, MIT with lymphoid cells derived from tumor bearing animals (Table 2) only showed a modest response as compared to the results obtained with lymphoid cells from healthy animals immunized with MMC treated tumor cells (Tables 3–5).

Our material includes a single spontaneous mammary tumor. Extracts of this tumor (T9) cross-reacted with anti-T3 lymphocytes in the migration inhibition test. Similar cross-reactions with unrelated tumors were reported by Cornain *et al.* [10] who found that this cross-reaction

could be eliminated by purification of the spleen cell population.

Fields *et al.* [12] raised 2 antisera in C3H mice against ENU induced neurogenic rat tumors. These antisera defined two cell surface antigens, i.e. a common antigen shared by rat brain, embryonic tissues and neurogenic tumors and a restricted antigen present in 6 tumors but not in normal adult or embryonic tissues.

Antigens cross-reacting with fetal antigens has previously been reported in human neurogenic tumors by Oda [13] and by Trouillas [14]. However, this was not confirmed by Dittmann *et al.* [15] who failed to demonstrate five fetal antigens present in human fetal brain, in human glioblastomas and meningiomas. Alpha-feto protein was also absent from these tumors. On the other hand, using rabbit antisera raised against human neurogenic tumors several antigens not present in fetal or adult brain were found. However, these antigens cross-reacted with antigens present in normal liver and were therefore not tumor specific.

In the present investigation the migration inhibition test revealed cross-reactions between extracts of fetal tissues and lymphoid cells sensitized to T1, T2 and T3 tumor cells. The most consistent results were obtained with anti-T3 lymphoid cells. In addition anti-T1, but not anti-T2 and T3, cross-reacted with extracts of normal adult brains.

These observations indicate that ENU induced neurogenic tumors share a common antigen which may be a fetal antigen. But it cannot be excluded that they share more than one common antigen, some of which may be tumor specific.

The expression of fetal antigenicity seems to reach its maximum during the second week of gestation [16]. Therefore, it is a little astonishing that anti-T1 lymphoid cells cross-reacted with fetal tissue at 1 week of gestation but not at 2 weeks. However, the difference between MII of sensitized and non-sensitized lymphoid cells exposed to 2-week-fetal brain was on the border-line of statistical significance and may possibly be substantiated on reinvestigation.

The T1 tumor also seems to share an antigen with adult brain. In contrast to the above mentioned observations by Fields *et al.* [12], our findings indicate that the cross-reaction with fetal tissues and adult brain depend upon at least two separate antigens.

No cross-reaction was found with adult liver. The cross-reaction with adult brain might therefore depend on the presence of nerve specific proteins in ENU induced neurogenic tumors. Even if S-100 was found to be present only in minimal amounts in human glioblastoma [15] it has been demonstrated in ENU induced neurogenic tumors [17–19] which also contain the 14–3–2 protein [20].

Moreover, human glioblastoma has been found to be rich in glial specific protein [15] and Bignami [21] has demonstrated GFA in organ cultures of human and ENU induced rat tumors.

Common antigens also could be of viral origin even in chemically induced tumors. Diwan *et al.* [22] found that ENU induced mouse leukemia and pulmonary lung adenomas were positive for both type-C RNA tumor virus and its group specific antigen. Denlinger *et al.* [23] reported the presence of viral articles in ENU induced neurogenic mouse tumors, and Lantos [24] discussed the origin and the significance of two intracytoplasmic virus like structures which he found in ENU induced rat gliomas.

Thus a number of observations indicate that chemically induced neurogenic tumors share one or several common antigens which generally seem to be rather weak and not tumor specific. The origin of these antigens is still unclear, but it does not seem unlikely that both fetal proteins and nerve specific proteins play a role. The individual specificities reported by some authors remain to be confirmed.

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Vincristine (NSC 67574) in Non-Small Cell Bronchogenic Carcinoma. Results of a Phase II Clinical Study

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Abstract—*A Phase* II *study of vincristine* (*NSC* 67574) *in pathologic categories* 1, 3 *and* 4 (*WHO*) *bronchogenic carcinoma was performed. Partial objective responses were observed in* 5 *of* 15 *patients with adenocarcinoma. Responses were observed in pulmonary masses* (5/15), *metastasic cervical nodes* (2/5) *and hepatic metastases* (1/2). *Median survival for patients presenting a response to VCR was* 339 *days, compared to* 73 *days for non-responders.*

Only 1 *of* 10 *patients with epidermoid carcinoma and none of* 10 *patients with anaplastic-large cell carcinomas responded to VCR therapy.*

It was concluded VCR is active in adenocarcinoma of lung and it might deserve further evaluation in Phase III *trials.*

INTRODUCTION

A REVIEW of current literature indicated that vincristine (NSC 67574) has not been adequately evaluated in bronchogenic carcinoma [1]. In a recent report, Hansen confirmed the early suggestions of activity of vincristine (VCR) in small cell bronchogenic carcinoma [2, 3]. In this study, partial objective responses were obtained in approximately one-third of patients resistant to a previous combination chemotherapy [4]. Since this report, VCR has been widely used in the intensive combination therapy of small cell carcinoma of the lung [5].

However, there appeared to be no available information on the effect of VCR in the therapy of the other pathologic types of lung cancer. This information can be considered important because VCR is a suitable drug for combination chemotherapy. Consequently, a Phase II study, to determine the activity of VCR, in epidermoid carcinoma, adenocarcinoma and anaplastic large cell carcinoma was designed. The objective of this report is to present the results obtained in this study.

MATERIAL AND METHODS

Patients were selected according to the following criteria: (1) Pathology proven bronchogenic carcinoma of the categories 1, 3 and 4, or epidermoid, adeno and anaplastic-large cell carcinomas, of the WHO [6]. (2) Advanced, non-resectable disease. (3) Measurable, previously untreated disease in the chest that could be easily followed in the chest X-ray. (4) Performance status above the 40% on the Karnofsky scale. (5) No previous chemotherapy. (6) Ability to continue out-patient follow-up. (7) Patients with marked senility or life expectancy of less than 3 weeks were excluded as well.

Eligible patients, stratified according to the tumor cell type received vincristine, 1.5 mg fixed dose in 200 cc 5% dextrose solution infusion over 4–6 hr. Vincristine was administered weekly for 10 weeks unless progression was documented earlier. After 10 weeks an evaluation was made and patients with stable disease or objective response were continued on the weekly dose until toxicity was noted at which time the drug was given every other week.

Neurologic toxicity was considered mild if there were tolerable paresthesias and loss of tendon reflexes. Moderate toxicity were painful paresthesias, disabling weakness, sensory loss and abdominal cramps. When a moderate neurotoxicity was noticed, VCR was discontinued for 2 weeks or one month and if improved during the interval it was again resumed at the same dose every other week.

The results were classified as objective response and progression. Objective responses were complete and partial. Complete response was defined when all evidences of measurable

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and non measurable disease disappeared and the patient remained free of disease for more than 1 month. Partial response was a decrease of more than 50% of the tumor size measured by the product of the two longest perpendicular diameters in all measurable disease with improvement in the non-measurable disease maintained for more than one month.

Progression was a decrease of less than 50% the tumor size, prolonged stable disease and progressive growth of the tumor.

All patients cases presenting a diffuse interstitial or alveolar pattern in the chest X-ray received a pretherapy course of antibiotics and/or digitalis before the proper baseline control films were taken.

RESULTS

VCR was given according to the study design to 35 previously untreated patients. There were 28 males and 7 females. Ages ranged from 44 to 76 yr, with a median age of 61 yr. Regarding to the performance status, 7 patients rated 40–50 in the Karnofsky scale, 17 patients rated 60–70 in the Karnofsky scale and 11 patients rated 80–100 in the Karnofsky scale. Eighteen patients had stage IV disease and 17 patients had stage III disease. Seven patients had received previous surgery or radiation therapy.

Response

Category 3 (*adenocarcinoma*). Five partial responses were observed among 15 patients with adenocarcinoma (Figs. 1–4). Objective responses were determined after 3–8 weeks of therapy. The median time to response was 6 weeks. The duration of responses ranged from 103 to 415 days. The objective response rate for this group was 33%. Some patients not qualifying for objective response showed some antitumor effect due to VCR, 2 patients presented less than 50% decrease in tumor size and 3 other patients presented decrease of pleural effusion or very prolonged stabilization of disease which progressed rapidly upon discontinuation of the drug after a certain period of time. These patients however were considered ineligible for objective partial response.

None of the patients presenting initial progression to VCR responded subsequently to a second drug therapy. One patient responding initially to VCR presented a second partial response to methotrexate for a period of 10 months.

Category 1 (*epidermoide carcinoma*). One partial objective response was observed among 10 patients with epidermoid carcinoma. This response was observed at the 4th week and lasted 175 days. Upon progression this patient received alkylating agent and presented a second objective partial response.

Only 1 of 9 patients in this category not responding to the initial treatment with VCR responded to a subsequent drug therapy.

Category 4 (*anaplastic-large cell carcinoma*). None of 10 patients in this category responded to the treatment with VCR. There were no subsequent responses to other drug therapies among this group of patients.

The influence of response upon survival can be seen in Table 1. Table 2 demonstrates the response rate for each tumor type according to the site of metastasis.

Toxicity

Moderate neurologic toxicity was usually evaluable in patients receiving VCR for 10 weeks. Seven of 11 patients treated with VCR for 10 weeks or more presented moderate toxicity; 3

Table 1. *Median survival time in days*

	Partial response	Progression
	(Number of patients)	
Epidermoid carcinoma	992 (1*)	72 (9)
Adenocarcinoma	339 (5)	73 (10)
Anaplastic carcinoma	—	80 (10)

*This patient presented a second objective partial response upon progression to VCR.

Table 2. *Responses according to site of metastases*

	(Responses/number of patients)				
	Lung	Lymph nodes	Skin	Liver	Bone
Epidermoid carcinoma	1/10	1/7	0/1	—	0/1
Adenocarcinoma	5/15	2/5	0/1	1/2	0/1
Large-cell carcinoma	0/10	0/4	0/3	0/3	0/1

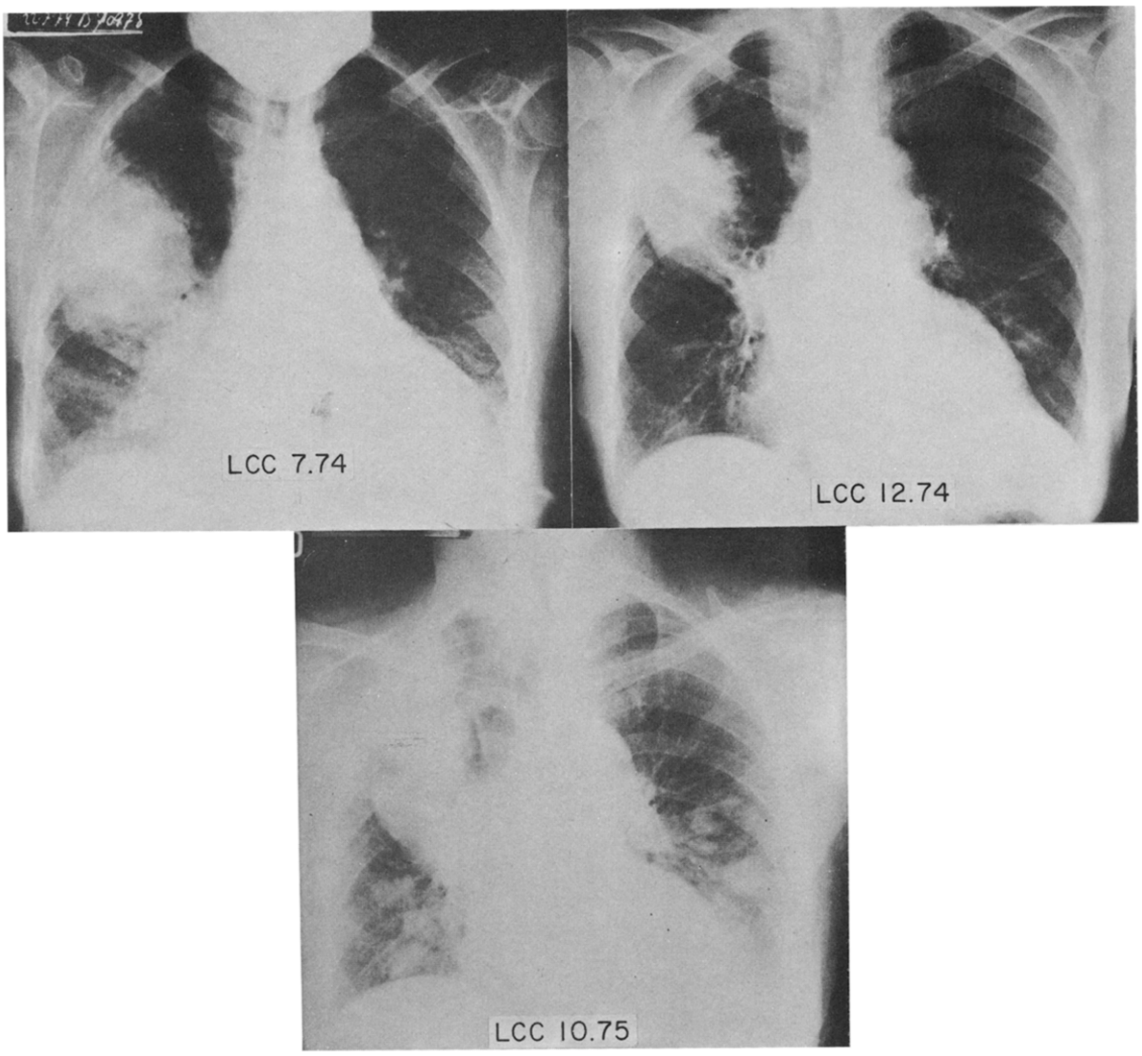

Fig. 1. Adenocarcinoma.

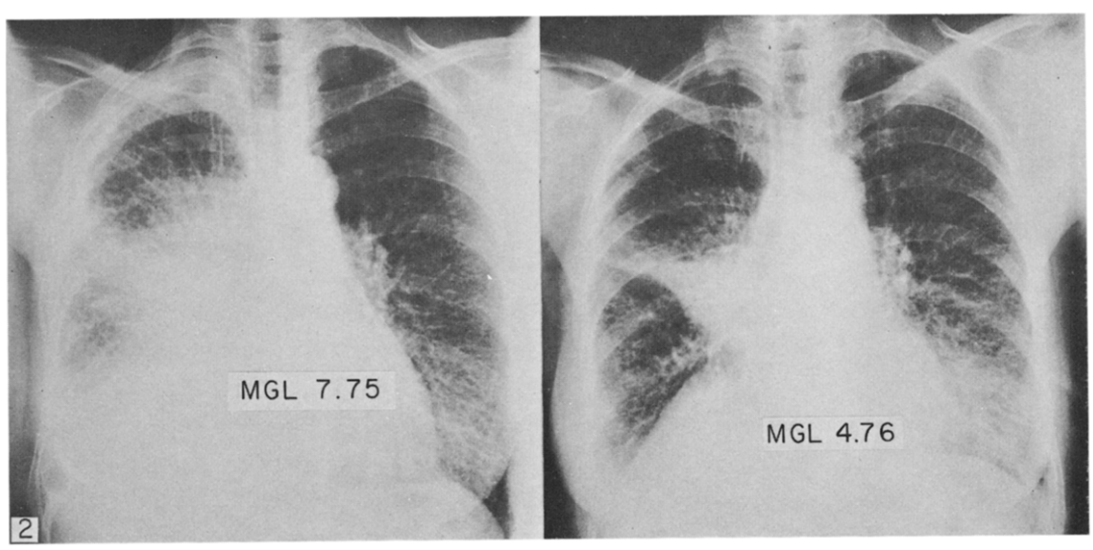

Fig. 2. Moderately differentiated adenocarcinoma.

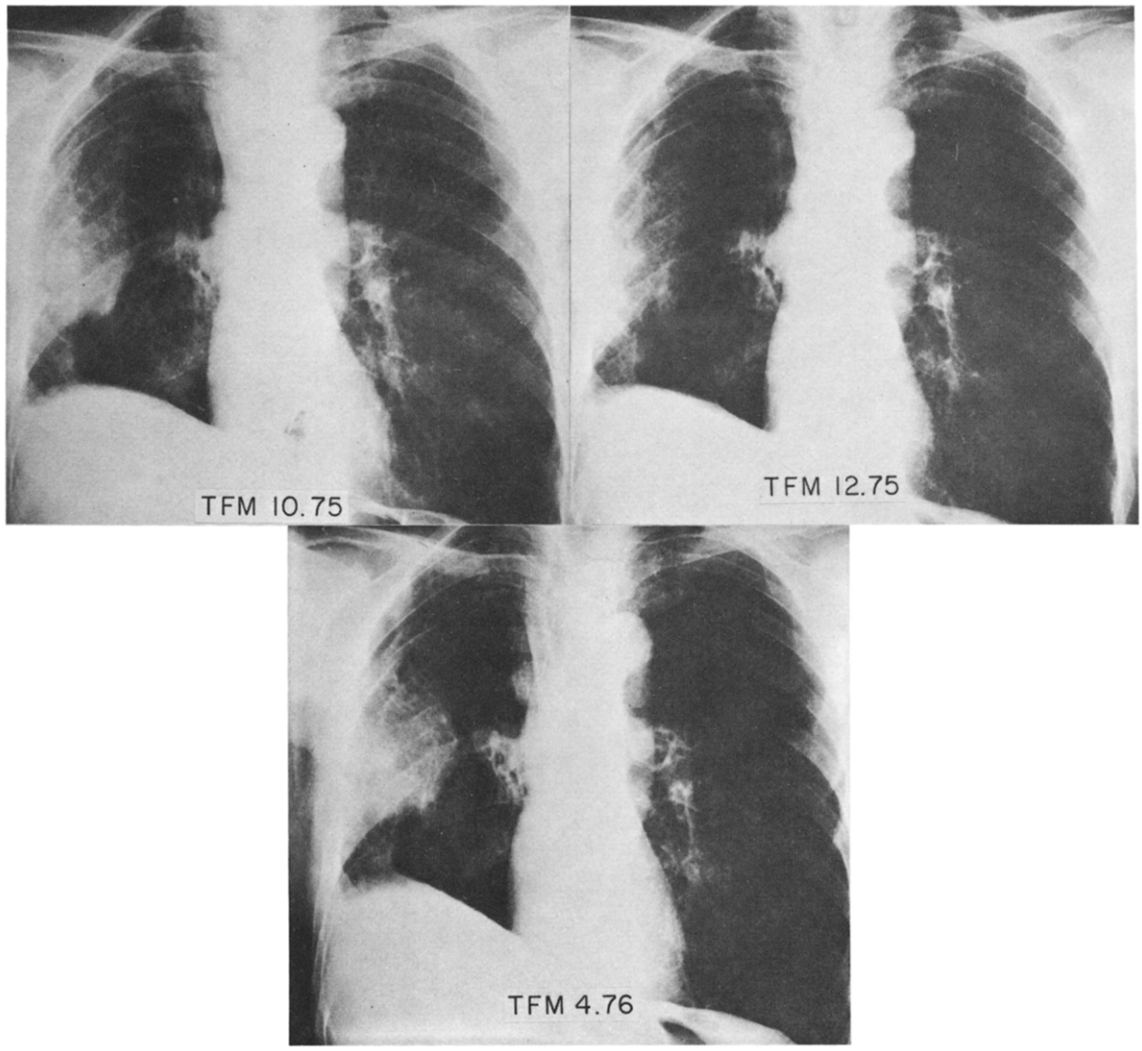

Fig. 3. Adenocarcinoma, well differentiated.

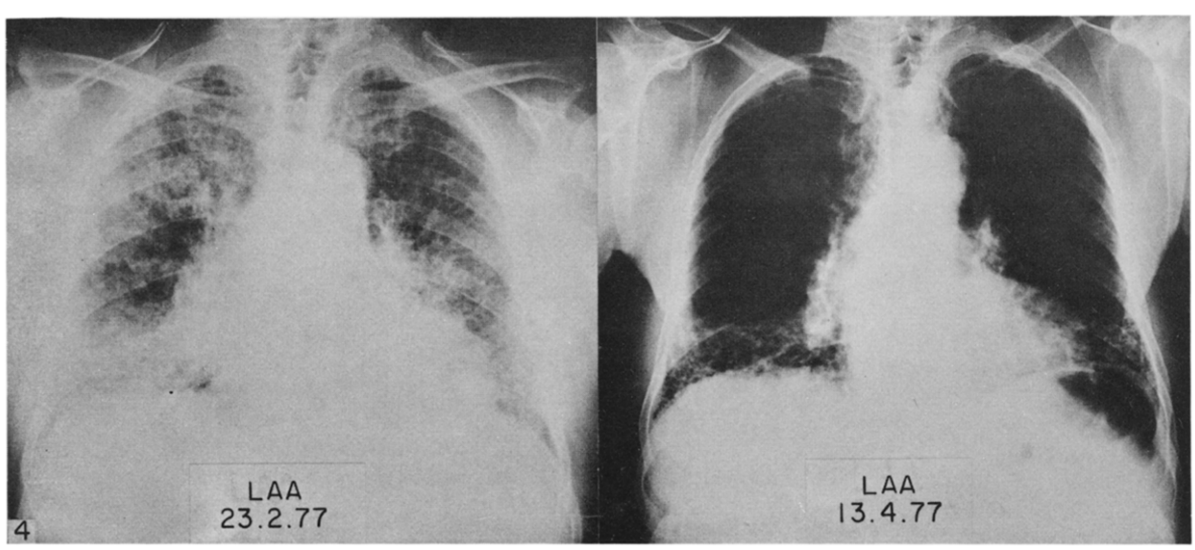

Fig. 4. Well differentiated adenocarcinoma, papillary type, bilateral.

with abdominal cramps or severe constipation and 4 with disabling weakness as the leading symptom. Four of these 7 patients continued VCR every other week after a period of rest of 2 weeks and appeared to tolerate it well. Two patients needed one month rest period and then continued receiving VCR every other week with good tolerance. One patient had VCR definitely stopped at his own request.

Mild neurologic toxicity was readily observed after 4 weeks of therapy. After discontinuation of the drug the symptoms regressed in 1–2 months.

No other clinically significative toxic manifestations could be attributed to VCR therapy in this study.

DISCUSSION

VCR appears to be active in adenocarcinoma of the lung, tumor relatively resistant to chemotherapy [1]. This activity has not been uncovered earlier because the systematic Phase II study design of the modern chemotherapy was not in use during the early sixties, when VCR was entering clinical trials. Otherwise, the activity of VCR in this cell type it is not completely unexpected since some activity is found in other adenocarcinomas such as breast cancer [7]. Further experiences using VCR in single or combination chemotherapy for bronchogenic adenocarcinoma remains a task for the large groups since this tumor type is relatively uncommon, and large number of patients are needed to establish firm conclusions.

In this study VCR appeared to have poor activity in epidermoid and anaplastic-large cell carcinoma.

The dosage of VCR used in this study is somewhat unorthodox. The reasons for using a low dose of Vincristine in this study were: (1) Dose-related neurotoxicity probably reflects the cumulative dose given. In this respect, it was postulated that a low dose schedule would delay the appearance of significant neurotoxicity allowing for a prolonged initial course in the majority of the patients. (2) One might hypothesize from a kinetic approach that in adult slow growing solid tumors it might be more important to schedule the drug administration: that is, frequency and length of the infusion, than the total amount of drug given per single dose. Consequently, emphasis was put on the weekly administration of a slow infusion.

Moreover, when one considers the total amount of drug administered in a given period of time, the long term optimal schedule of VCR used as a single agent it is probably unknown and it has been seldom utilized [8]. (3) And finally, it was thought that if the most important single prognostic factor of survival in lung cancer is the performance status of the patient, neurotoxicity should be avoided or delayed because it could have a direct bearing in the performance status quality of the patients. In this regard it was postulated that a weekly standard dose of VCR could produce unacceptable toxicity and a low dose could be more suitable.

In restrospective analysis, the dose schedule used proved to be adequate in the induction and maintenance of the responses obtained. This might have had a particular interest since some patients could be carried on the VCR infusions for over 1 yr with a near normal active life.

It was concluded that VCR is active in bronchogenic adenocarcinoma and further trials, possibly of combination chemotherapy with other active agents, should be conducted.

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Pharmaco-Kinetics of Melphalan Following Oral or Intravenous Administration in Patients with Malignant Disease*

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Abstract—*The distribution and excretion of* C^{14}*-labelled melphalan has been determined in seven patients with disseminated malignant disease. Following intravenous administration, melphalan was distributed in the total body water and disappeared with half-lives of approximately* 67 min *and* 160 hr. *Up to* 65% *of label was recovered in the urine over a period of seven days following intravenous administration. Following oral melphalan, peak blood levels were seen at* 2 hr; 30% *of the administered label was recovered in the urine over* 9 *days and* 20–50% *of administered label in faeces over* 6 *days. These results show that absorption following oral melphalan treatment is incomplete and that the drug has a prolonged terminal plasma half-life. Drug metabolism is not extensive and the majority of label recovered from plasma and urine taken within* 5 *and* 40 *min of i.v. drug administration cochromatographed with melphalan.*

INTRODUCTION

MELPHALAN was introduced into clinical use in the late 1950's and has since established itself as an agent with a wide spectrum of antitumour activity. It is used extensively in the treatment of ovarian cancer and myeloma and as adjuvant therapy in breast cancer [1–3]. Tables are the most commonly used preparation but, in some situations, i.v. melphalan is preferred.

Although melphalan has been used clinically for nearly 20 yr, there is little information on its clinical pharmacology. [4] We now report pharmaco-kinetic studies of labelled melphalan in 7 patients with disseminated malignant disease. In 4 patients, melphalan was given as an i.v. injection. In the others, the drug was given orally—in 2 patients on the first day of a 5-day course of melphalan treatment and, in the third patient, on the 3rd day of a 5-day course of treatment.

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MATERIAL AND METHODS

Universally C^{14}-labelled melphalan (3.6 mCi/nmole) was supplied by the Radiochemical Centre, Amersham, England and C^{14}-labelled melphalan in tablet form (20 mCi/1.9 mg) was prepared from this material. Prior to i.v. administration, the identity and purity of the C^{14}-labelled melphalan were confirmed by chromatographic analysis. The i.v. solution was sterilized by millipore filtration and then mixed with unlabelled melphalan to give the required total dose. Unlabelled melphalan tablets were given with the labelled tablet to make up the appropriate dose.

Clinical details

Details of the 7 patients studied are shown in Table 1. Patients received either a single intravenous injection of melphalan (20–23 mg/m^2, 14 mCi) or 20 mCi of labelled melphalan was given orally during a 5-day course of oral melphalan treatment (4.4–6.4 mg/m^2/day). In 2 patients (JM and BS), labelled drug was given with the first oral dose of melphalan, while JT received 2 days of unlabelled melphalan and then the labelled compound.

Table 1. *Patient details*

Patient	Sex	Tumour	Age	Melphalan dose (mg)	Route	Surface area
M.D.	F	Melanoma	63	38	IV	1.7
W.T.	M	Adenoca. colon	70	40	IV	1.9
A.L.	F	Adenoca. pancreas	59	38	IV	1.65
J.S.	F	Melanoma	40	31	IV	
*J.M.	M	Melanoma	64	9 (× 5d.)	0	1.5
†J.T.	F	Myeloma	70	10 (× 5d.)	0	2.3
B.S.	M	Adenoca. colon	76	10 (× 5d.)	0	1.5

*Labelled melphalan given on first day of course.
†Labelled melphalan given on third day of course.

Renal function was normal in all patients but hepatic function in one was severely deranged. In this patient (AL), bilirubin levels were falling rapidly, following a cholecystojejunostomy for treatment of carcinoma of the head of the pancreas.

At regular intervals, following the administration of labelled melphalan, blood was drawn and plasma separated. All urine and stool specimens were collected for periods up to 9 days following labelled melphalan treatment and aliquots were taken for counting. Specimens were stored at −30°C until analysis.

Extracting and counting procedures

One half millilitre aliquots of plasma were suspended in 10 ml of monophase 40 and counted in a liquid scintillation counter. One to two millilitre aliquots of plasma were ultrafiltered at 1000 ***g*** through amicon membrane cones (type CS50A), which exclude greater than 97% of plasma proteins and 0.5 ml aliquots of the ultrafiltrate were counted in monophase 40. Lymphocyte and red cells were completely combusted to carbon dioxide and water in a Packard tricarb sample oxidizer. The carbon dioxide was collected in 10 ml of carbosorb and permafluor V and counted for 10 min. Aliquots of stool samples were combusted in a similar manner and counted. Aliquots of the 24-hr urine specimens were centrifuged to remove solids and 0.5 ml of the supernatant counted for 10 min in 10 ml of monophase 40. On patient JS the concentration of unmetabolised melphalan in the first two urine samples was measured directly, using mass spectrometry. C^{14}-labelled carbon dioxide in expired air was measured daily following labelled drug administration and, after collection of one mole of carbon dioxide in a carbon dioxide absorbant with a phenolphthalein indicator, the absorbant was counted.

Quantification of Melphalan in plasma and urine (Patient JS)

Plasma and urine samples were collected from one patient and metabolites of melphalan were sought.

Plasma samples. Fifty microlitres of melphalan solution (5 mg per 5 ml in 0.1 N HCl) was added to 1 ml plasma. This was added to 10 ml of a 1 : 2 mixture of acetone: ethanol and shaken for 15 sec before brief centrifugation. The supernatant was decanted and evaporated to dryness. The concentrate was dissolved in 100 µl of water and 100 µl of this solution was spotted on a silgur plate and run overnight in butanol : water 86 : 14.

Urine samples. One hundred microlitres of urine were added to 50 µl of melphalan solution (5 mg per 5 ml) and 100 µl of the resultant solution spotted on a silgur plate and run overnight in butanol : water 86 : 14.

All plates were scraped in 1 cm bands into counting vials, stood overnight with 0.5 ml HCl (N/10) and counted after dilution with 10 ml scintillation fluid (naphthalene, POP and POPOP in ethanol, dioxan and toluene) on a Packard Tricarb 3320 scintillation counter. In addition, the concentration of melphalan in the first two urine samples (0–40 min and 40–110 min) was measured directly as follows: 1 ml sample was taken and 20 µl melphalan-d_2 (1 mg/ml in 0.1 N HCl) as internal standard was added. The sample was adsorbed on to an XAD 2-column (8 × 1 cm)® and washed with 25 ml distilled water, which was discarded. The melphalan was eluted with 10 ml CH_3OH and the eluate concentrated to dryness. The concentrate was taken up in 0.5 ml CH_3OH and allowed to stand with 0.5 ml ethereal diazomethane for 10 min at room temperature, then concentrated to dryness, taken up in methanol and spotted on to a fluorescent silica thin-layer chromatographic plate which was developed in

chloroform : methanol 19 : 1 alongside a plate spotted with melphalan which had been similarly methylated. The melphalan methyl ester (R_F 0.55) was eluted with methanol and the ratio of melphalan to deuterated melphalan in the sample determined by the ratio of peaks at m/e 230–232 $(M\text{-}CH(NH_2)CO_2Me)^+$ for d_0-d_2-ester) on mass spectrometry.

Mass spectrometry

Mass spectra were determined using an AEI-MS12 spectrometer after concentration of methanol solutions onto the direct insertion probe. The ionizing voltage was 70 eV, trap current 100μA and ion-source temperature *ca.* 120°C. For determination of the ratio of melphalan : melphalan-d_2 in samples, spectra were scanned repetitively at 10 sec/decade. The intensities of peaks in the region containing the ions of interest (m/e 230, 232) were recorded using an Instem Maxi data system. The average of about 10 scans was taken. For reduction of observed peak intensities to give the molar ratio of melphalan : melphalan-d_2, correction factors were applied for:

(a) The contribution to m/e 232 (base peak for d_2-analogue) of the $^{37}Cl^{35}Cl$-containing ion corresponding to m/e 230 which is present in the mass spectrum of the d_0-analogue;

(b) The contribution to m/e 230 due to the small percentage (1.25%) of non-deuterated melphalan present in the d_2-standard.

A linear relationship, after applying these correction factors, was demonstrated between the composition of mixtures of synthetic d_0 and d_2-forms and the corrected peak intensities.

Synthesis of melphalan-d_2 standard

A solution of melphalan (0.2 g) in deuterium chloride in D_2O (20% w/v, 1 ml) was heated under reflux for 20 min. The cooled solution was diluted with methanol and applied to a column (22 × 2 cm) of amberlite IR45 ion-exchange resin which had previously been washed with methanol. The product was eluted with 200 ml methanol and was recrystallised from this solvent (2 ml) to yield melphalan-d_2 (20 mg) as a white solid.

The mass spectrum showed a molecular ion $(M^{+\cdot})$ at m/e 306 (3% rel. int.) and a base peak at m/e 232 $(M\text{-}CH(NH_2)CO_2H)^+$. From the ratio m/e 232 : m/e 231 : m/e 230 the product contained the d_2, d_1 and d_0 forms in the ratio 100 : 5.0 : 1.25.

By analogy with the corresponding conversion of aniline mustard® into a d_3-analogue, melphalan is probably deuterated at the positions *ortho* to the N-mustard substituent.

Thin layer chromatography

For assay by radioactivity, silgur-25UV$_{254}$ plates (Camlab Ltd., Cambridge), 20 × 20 cm were used. Samples were applied as 3 cm-wide strips to the Kieselguhr zone of these plates. U.V.

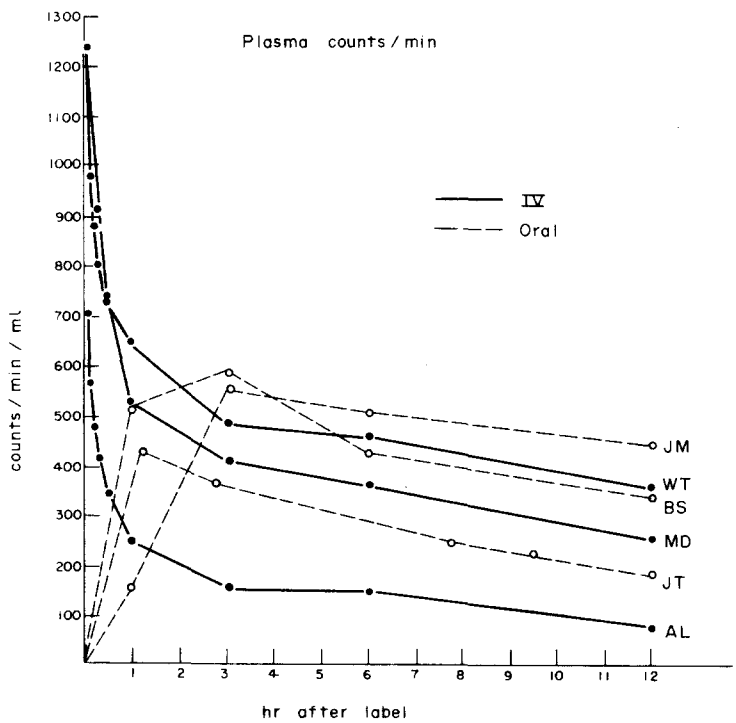

Fig. 1. Plasma pharmacokinetics of labelled melphalan administered to six patients with malignant disease.

absorbing bands were located using a Hanovia chromatolite. For thin-layer chromatography of melphalan methyl ester prior to mass spectrometry, glass plates (20 × 5 cm) coated with Merck Kieselgel GF $_{254}$ were used.

RESULTS

Figure 1 shows the plasma levels of melphalan with time following the administration of melphalan to six patients. Plasma concentrations of melphalan in JS are presented in Table 2 and Fig. 2. Extrapolation of the initial linear portion of the curve to $t=0$ gives a theoretical volume of distribution of 44.3 litres and a plasma $t\frac{1}{2}$ for the drug of 67 min. It is clear that there is a prolonged terminal phase in the disappearance of label from the plasma as demonstrated in Figs. 1 and 3 and the half-time approximates 160 hr. Following oral administration, peak label occurred in plasma at 2 hr and then declined with a similar half-life to the i.v.-treated patients (Figs. 1 and 3).

Table 3 shows the percentage of plasma counts retained on plasma protein after filtration. In all patients studied except AL, over 50% of the counts were retained. The percentage of counts retained increased with time following treatment and over 90% of the plasma counts were unfilterable after 12 hr..

AL was markedly icteric at the time of melphalan treatment and studies were undertaken with plasma from other jaundiced patients. Preliminary studies indicate that jaundice serum binds melphalan less well than normal serum (data not presented).

Figure 4 shows the cumulative excretion of labelled melphalan in the urine and stool of 4

Table 2. Plasma concentrations of melphalan following i.v. administration of 31 mg (patient J.S.)

Time (min)	Melphalan (μg/ml)
5	1.53
10	0.97
30	0.60
65	0.39
180	0.11

Table 3. Plasma protein binding of labelled melphalan

Time	% of total counts retained on Amicon filter Patient				
	W.T.	A.L.	J.T.	J.M.	B.S.
5 (min)	68	0	—	—	—
60	66	1	67	54	64
360	—	—	—	57	72
540	—	—	85	—	—
720	87	28	—	65	83
1 (days)	—	—	92	84	85
3	—	—	—	86	94
4	—	—	97	91	92
5	91	89	—	—	—
7	95	—	—	—	—
12	—	97	—	—	—

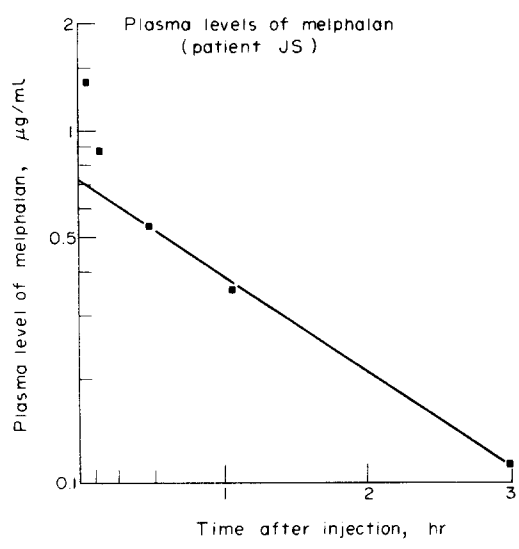

Fig. 2. The initial plasma pharmacokinetics following i.v. administration of labelled melphalan (patient J.S).

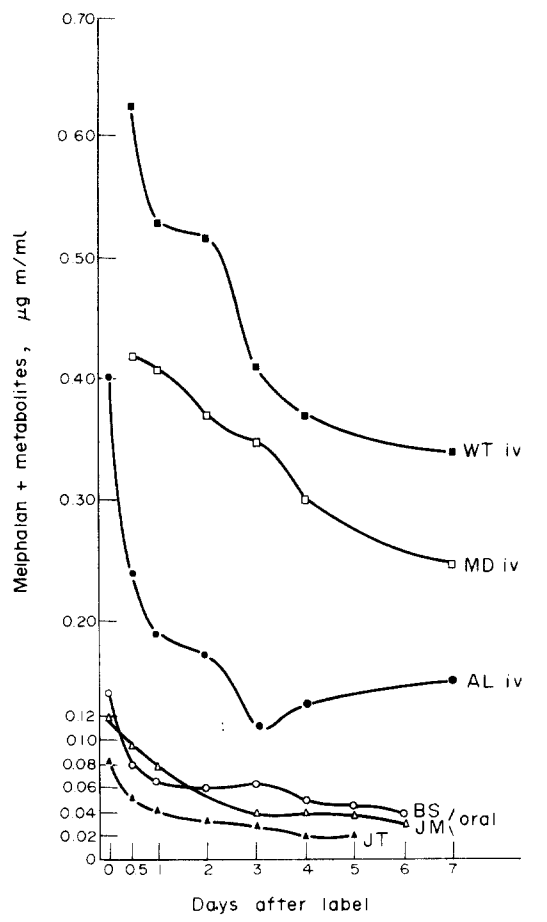

Fig. 3. Plasma pharmacokinetics of labelled melphalan—terminal disappearance curves in 6 patients with malignant disease.

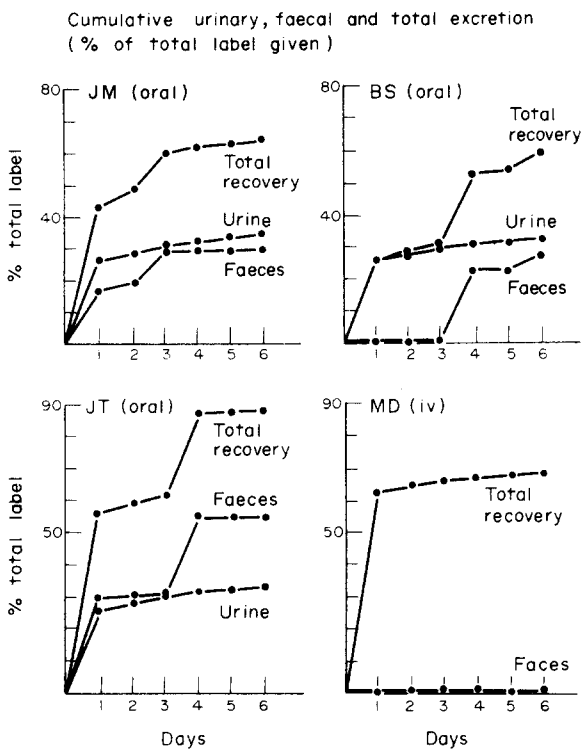

Fig. 4. Cumulative urinary, faecal and total excretion of labelled melphalan in 4 patients.

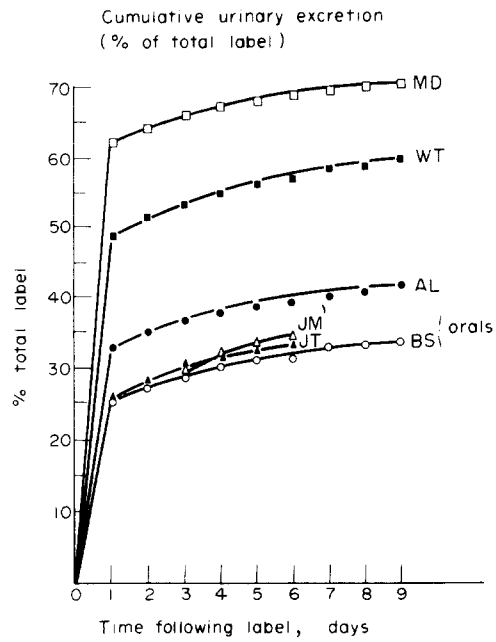

Fig. 5. Cumulative urinary excretion of labelled melphalan in 6 patients.

Table 4. Excretion of melphalan in urine (μg/ml) *following i.v. administration* (31 mg)

Assay method	Time (min)			
	40	110	240	420
Radioactivity	108	37	1.8	1.0
Mass spectrometry	95.9	34		

patients on whom data are available. The results indicate that only small amounts of label are recoverable in the stool following i.v. melphalan treatment and, in the 1 patient with complete data, approximately 60% was recovered in the urine 24-hr after administration—with only a small amount of further label being recovered over the next 5 days. Throughout this period, faecal recovery was minimal. In 3 patients receiving labelled melphalan orally, between 20 and 50% of the label was recovered in the faeces over 6 days.

Figure 5 shows cumulative urinary excretion of label with time in 6 patients. The patients who received labelled melphalan orally excreted substantially less label in their urine than those patients receiving the drug i.v. Approximately 30% of the administered label was recovered in the urine in the oral group over a period of 6 days while, in the i.v. group, urine recoveries varied betwen 35 and 65%. In each case, the pattern of label recovered was very similar, with the majority being recovered in the first 24 hr following treatment. There was no suggestion that prior loading with melphalan (patient JL) altered the pattern of recovery.

Table 4 shows the level of melphalan recovered in the urine of patient JS following intravenous administration. The results indicate that the levels of melphalan measured by mass spectrometry or by calculation from radioactivity are very similar.

No labelled carbon dioxide was measured in expired air.

Metabolite studies

Figures 6 and 7 are histograms of the chromatography plates for a 5-min plasma sample and 40-min urine sample from patient JS. The principal radioactive peak in both cases was exactly coincident with the u.v. detectable melphalan band. These results indicate that major metabolism of the labelled melphalan had not taken place at this time.

DISCUSSION

This is the first study of the clinical pharmacology of melphalan given both orally and intravenously. The results indicate that there is incomplete absorption of melphalan given by mouth. This finding raises the possibility that a standard oral dosage regimen, such as is used widely as adjuvant treatment of operable breast cancer, may be absorbed erratically and, in some patients, adequate blood levels of Melphalan may not be reached. These results do not identify

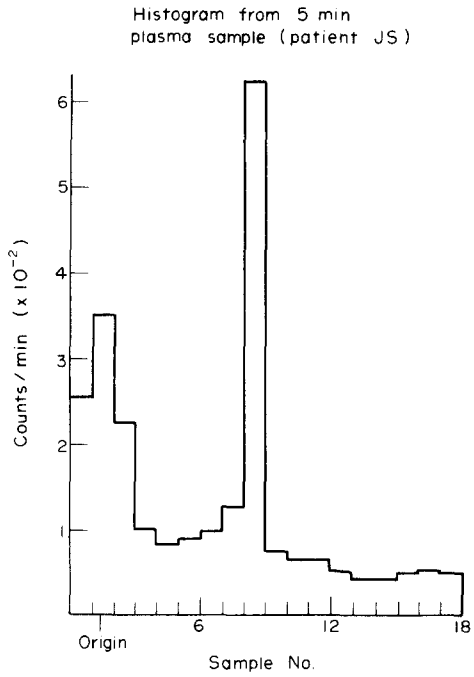

Fig. 6. Chromatogram of labelled melphalan extracted from plasma 5 min after drug administration (patient J.S.).

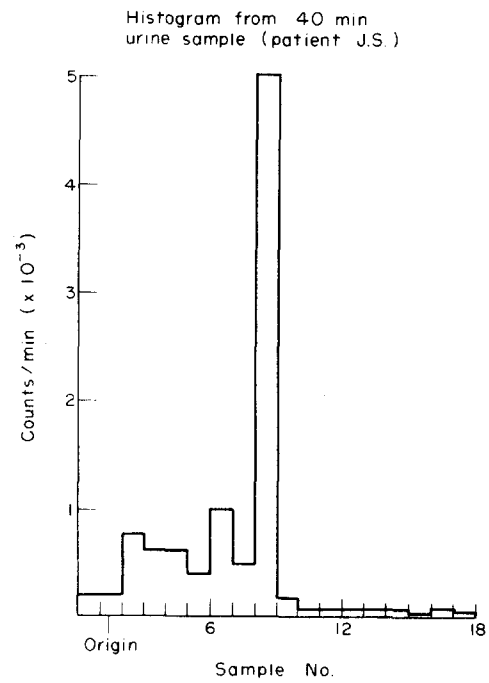

Fig. 7. Chromatogram of labelled melphalan extracted from urine passed 40 min after drug administration (patient J.S.).

factors influencing melphalan absorption and more detailed study may lead to improved understanding of these variables. At present, it would seem unwise to use a standard dosage of oral melphalan but rather to increase the dose with each course until myelosuppression is seen.

Melphalan given i.v. is distributed rapidly in the total body water and then disappears from the blood with a biphasic pattern. Following i.v. injection, 50% of the administered melphalan is recoverable in the urine within 24 hr. The plasma protein binding studies suggest that immediately after i.v. administration melphalan is only partially bound to plasma proteins but, with increasing time (and lower absolute melphalan concentrations), the proportion of drug associated with plasma proteins increases. The observation that hyperbilirubinaemic plasma bound less melphalan than normal plasma requires further study, but it indicates that competition may occur between melphalan and other drugs for plasma proteinbinding sites.

Following i.v. administration, melphalan is not excreted in the stool, while up to 50% of orally-administered drug may be recovered in the stool. The latter patients excreted less melphalan in the urine but the total recovery was higher than in the patient given melphalan intravenously.

Our studies of blood and urine collected within 5 and 40 min respectively of drug administration do not suggest that melphalan is metabolised significantly. The finding contrasts with the studies of melphalan pharmacology in dogs [5]. The general pattern of melphalan distribution in man and dogs appears to be similar but, in dogs, there is evidence of 6 chromatographically different products in extracts of different tissues. It is not known if such metabolites are present in human tissues. It is known that melphalan is not stable in aqueous solutions and it may be difficult to distinguish metabolic degradation of melphalan from physical instability. Nevertheless, our results on specimens taken soon after i.v. administration do not indicate significant metabolism.

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The Effect of Syngeneic Lymphoid Tumors Upon Mouse B-Lymphocyte and Granulocyte–Macrophage Colony Forming Cells*

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Abstract—*Granulocyte–macrophage (GM-CFC) and B-lymphocyte colony forming cell (BL-CFC) numbers have been determined in the lymph nodes, spleen and bone marrows of mice bearing either subcutaneous B or T lymphomas, or a mammary carcinoma. During the exponential period of tumor growth, all animals had increased levels of BL-CFC in the regional lymph nodes and spleen irrespective of the type of tumor. Mesenteric lymph nodes were unaffected by subcutaneous tumor growth.*

Tumors of B lymphocyte origin did not specifically influence levels of host BL-CFC. In the late plateau phase of tumor growth only the regional lymph nodes of the mammary carcinoma-bearing mice showed increased levels of BL-CFC whereas the incidence of BL-CFC was normal or subnormal in regional lymph nodes and spleen of the lymphoma transplanted mice.

The incidence of GM-CFC increased 5–200 times in spleen and bone marrow of mice transplanted with B lymphoma, plasmacytoma or carcinoma cells, whereas the growth of T lymphoma cells did not influence the levels of GM-CFC. Serum obtained from B lymphoma or plasmacytoma-bearing mice in the late period of tumor growth demonstrated a lack of inhibitory activity when compared with normal mouse serum or serum harvested in the early period of tumor growth.

The proliferation of B lymphocyte and granulocyte–macrophage precursors in mice transplanted with syngeneic tumors during the exponential period of tumor growth may represent a host protective response against tumor cells.

INTRODUCTION

HAEMOPOIETIC changes have been shown to occur in animals bearing transplantable tumors [1, 2, 3]. The dynamics of haemopoietic and lymphoid precursor cells of tumor bearing animals may be of importance to the understanding of the host-tumor balance, therefore we have examined these precursor cells in mice inoculated with syngeneic B or T lymphoma cells, or with carcinoma cells.

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Abbreviations used: BL-CFC = B-lymphocyte colony-forming cells. GM-CFC = Granulocyte–macrophage colony-forming cells. GM-colonies = Granulocyte-macrophage colonies. EBSS = Eisen's balanced salt solution. BM = Bone marrow. DME = Dulbecco's modified Eagles medium. 2-ME = 2-mercaptoethanol. SRBC = Sheep red blood cells. GM-CSF = Granulocyte-macrophage colony stimulating factor.

Fluctuations in B-lymphocyte colony-forming cells (BL-CFC) and granulocyte–macrophage colony forming cells (GM-CFC) have been examined in lymphoid and haemopoietic organs of tumor-bearing mice, using a semi-solid agar cloning system developed in this laboratory [4, 5]. The extent of tumor-infiltration of host lymphoid organs has been determined from numbers of tumor-colonies growing in the agar-cultures. Finally, the effect of serum from tumor-bearing mice on the cloning efficiency in agar-medium of the four tumor-lines employed has been studied.

MATERIAL AND METHODS

Animals

Inbred female BALB/c mice, 2–3 months of age were obtained from the Institute stock.

Tumor lines

All tumor lines studied were derived from BALB/c mice. They were maintained and

generously provided by Drs. A. Harris and N. Warner. The tumor cells used were obtained from continuous cell lines cultured as described by Harris *et al.* [6]. The following four tumor lines were used in the present work: ABLS-8, an Abelson virus induced early B-lymphocyte tumor [7]; P3K, a mineral oil-induced plasma cell tumor; WEHI-7, a T lymphoma induced as a thymic tumor by fractionated irradiation [6]; and EMT-6, a spontaneous breast carcinoma [8].

Tumor cells were washed twice and inoculated subcutaneously in the right flank as 10^7 cells in 0.2 ml saline.

Preparation of cell suspensions

At various periods of time after inoculation of tumor cells (or 0.2 ml saline i.e. controls) groups of three mice were bled for serum preparations and killed. The right axillary lymph nodes, the spleen, the mesenteric lymph nodes and the subcutaneously growing tumor were removed from each mouse and in some cases weighed. Single cell suspensions were made of pools of similar organs by teasing the organs with fine needles in 1–2 ml Eisen's balanced salt solution (EBSS). Bone marrow (BM) cells were flushed from the femoral cavity using 5 ml EBSS to 6 femora. A viable cell count was determined for all cell suspensions using eosin dye exclusion and the suspensions diluted in EBSS to the required cell number.

Culture technique

The general techniques for colony formation by B lymphocyte and granulocyte–macrophage precursors have been described in detail elsewhere [4, 5, 9]. Briefly, cells in vol of 0.2 ml were added to an equal vol mixture of double strength Dulbecco's modified Eagle's medium (DME) and 0.6% Difco Bacto agar. B lymphocyte cultures contained 5×10^{-5}M 2-mercaptoethanol (2-ME) and 0.05 ml 60% washed SRBC per 1 ml of agar-medium. Granulocyte and macrophage colony formation was stimulated by 0.1 ml of mouse lung conditioned medium (GM-CSF) [10] per 1 ml culture. The influence of serum on tumor colony formation was tested at various dilutions in 0.1 ml added to the empty culture dish prior to the addition of the agar-medium containing the tumor cells. All cultures were incubated in 33 mm plastic dishes at 37°C in fully humidified atmospheric air containing 10% CO_2 for a period of 6–7 days. Colonies (50 cells) were scored with an Olympus dissection microscope. Granulocyte–macrophage colonies were removed with a fine Pasteur pipette, placed on a microscope slide, allowed to air dry. After staining with aceto-orcein, colony morphology was determined according to previously published criteria [11]. The agar-medium for cloning of tumor cells was identical to that used for BL-CFC.

Identification of tumor cells in lymphatic organs and BM

All cultures for BL-CFC were set up in quadruplicate with and without 2-ME in the agar-medium. Since the WEHI-7, the P3K and the EMT-6 tumor cells grew independently of the addition of 2-ME, as few as 1 tumor colony forming cell per 25,000 lymph node or spleen cells could be detected by comparing the cultures with and without 2-ME. ABLS-8 tumor cells were dependent on addition of 2-ME but these tumor-colonies had a typical morphology, enabling them to be easily distinguished from B lymphocyte colonies [12].

Mixing experiments

To examine the influence on B-lymphocyte and GM-colony-formation of tumor colony proliferation constant numbers of normal spleen or BM cells (10,000 and 50,000/ml respectively) were mixed with varying numbers of tumor cells prior to agar seeding. The number of developing B-lymphocyte and GM-colonies was determined as outlined above.

Correction of mean colony counts

Individual data were normalized as follows: the average colony count of the same organ from 15 individual control groups was calculated (A). Normalized mean colony counts for the same organs in an individual group of tumor bearing mice were calculated as $A \times B/C$ where B represented the mean colony count of the experimental organ-pool and C represented the mean colony count in the corresponding control organ pool. All BL-CFC and GM-CFC-counts were corrected in this manner.

RESULTS

Tumor growth

Subcutaneous injection of 10^7 ABLS-8, P3K, WEHI-7, or EMT-6 tumor cells killed the recipient mice within a period of 3–4 weeks. Figure 1 shows the growth curves for the four tumor types. At the time of death, individual tumors weighed from 4 to 8 g.

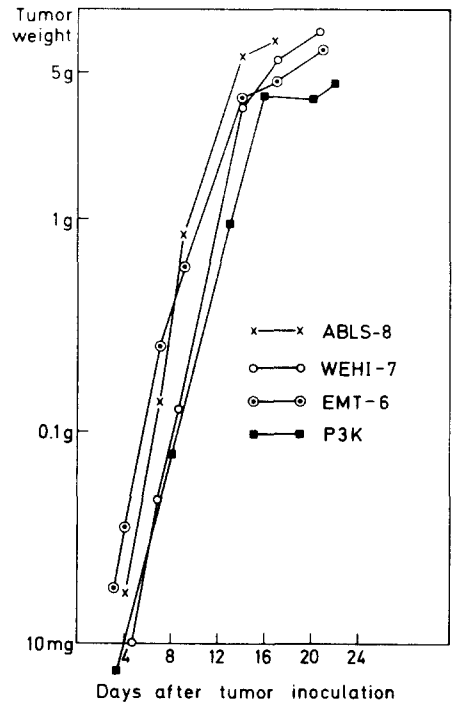

Fig. 1. In vivo *growth curves for the four tumor lines studied.* 10^7 *tumor cells were inoculated at* day 0. *Groups of* 3 *mice were killed at intervals as indicated. Data represent the mean tumor weight per animal.*

Lymph node and spleen weights

The regional axillary lymph node and spleen weights for groups of mice inoculated with 10^7 tumor cells are shown in Fig. 2. These data do not include those organs with extensive tumor cell infiltration (see below). In mice injected with ABLS-8 and P3K tumor cells, the spleen and lymph node weights increased only by a factor of 1.2–2.0, whereas T lymphoma cells did not influence spleen weight and only caused a slight weight increase in the axillary lymph nodes (Fig. 2). However, mice injected with carcinoma cells (EMT-6) showed weight increases in axillary lymph nodes and spleen by a factor of 4–6 regardless of the fact that very few tumor cells could be detected in the respective lymphoid organs (see below). Mesenteric lymph node weights were not influenced by tumor proliferation with any of the tumors tested.

BL-CFC in regional lymph nodes and spleen

The corrected mean values for BL-CFC in the regional axillary lymph nodes and the spleens of mice at different days after injection of tumor cells are shown in Figs. 3–4. Also shown are the numbers of tumor-colonies present in these organs. It is seen that only very few tumor cells were present in the cultured lymphoid organ suspensions, even during the latter part of the experimental period. Figures 3–4 do not include data from 3 groups of WEHI-7 tumor bearing mice (day 17, 21 and 22) and one group injected with ABLS-8 tumor cells (day 17). These 4 groups showed enormous enlargement of the axillary lymph nodes and spleen, the cultures being crowded with tumor colonies (i.e. 1000–

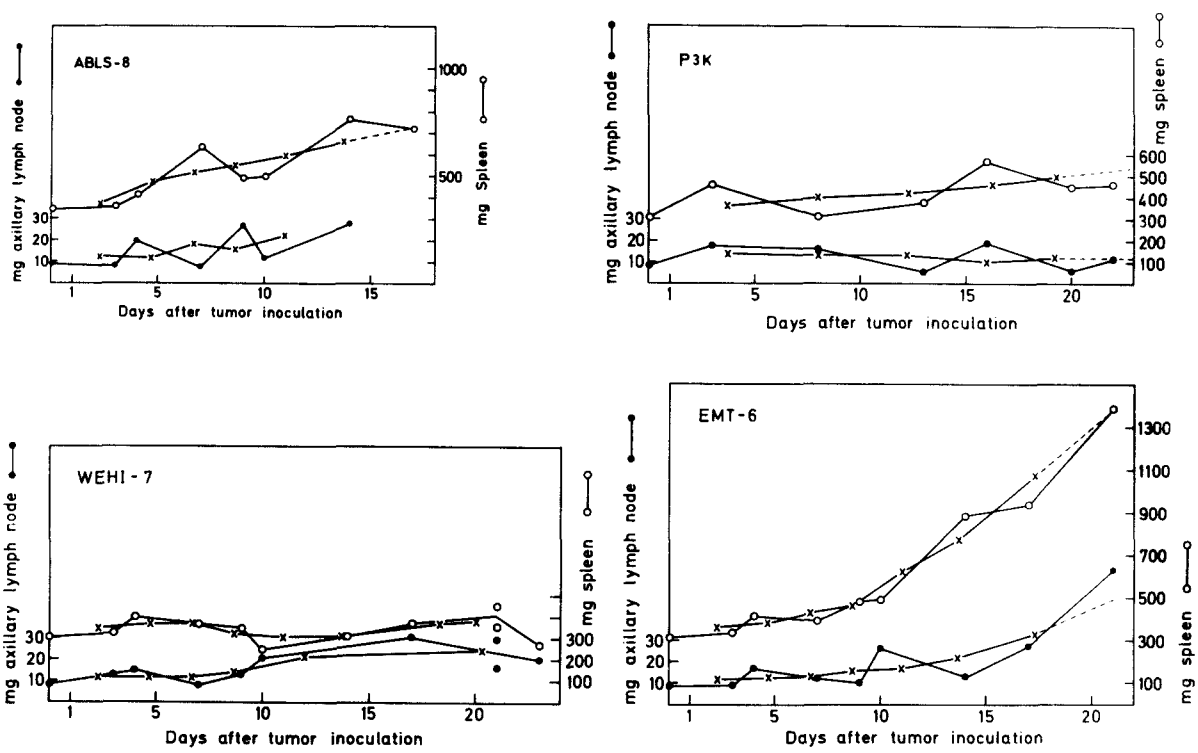

Fig. 2. *Weight curves of the axillary regional lymph nodes and the spleen of tumor bearing mice. Data represent weights of organs from groups of three animals. Data were fitted according to the least squares method* (× ——— ×).

5000 tumor colonies per 25,000 cells plated), making it impossible to determine the actual number of B lymphocyte colonies. With all types of tumor hosts there was an initial increase in the frequency and the total number of BL-CFC by a factor of 2–5. This increase was followed by a decline in frequency of BL-CFC, either below control values (the P3K tumor host) or to levels close to control values (WEHI-7 and ABLS-8 tumor hosts). For the EMT-6 bearing mice the frequency of BL-CFC decreased with time in the spleen whereas it increased in the regional lymph nodes. Except for the P3K hosts, there was an increase in the total number of lymph node and spleen BL-CFC in the latter phase of the tumor growth period partly due to the increased size of the organs.

The low frequency of tumor colonies detected in the regional lymph nodes and spleen, taken together with the very high cloning efficiency of these tumor cells (cf. Table 2), indicates that the lymphoid cell suspensions in the late period of tumor growth contained at most 2–5% tumor cells.

Bone marrow cellularity

Table 1 shows the total numbers of bone marrow cells in mice bearing each of the four tumor types studied. An influence on cell numbers (depression) was only observed in mice

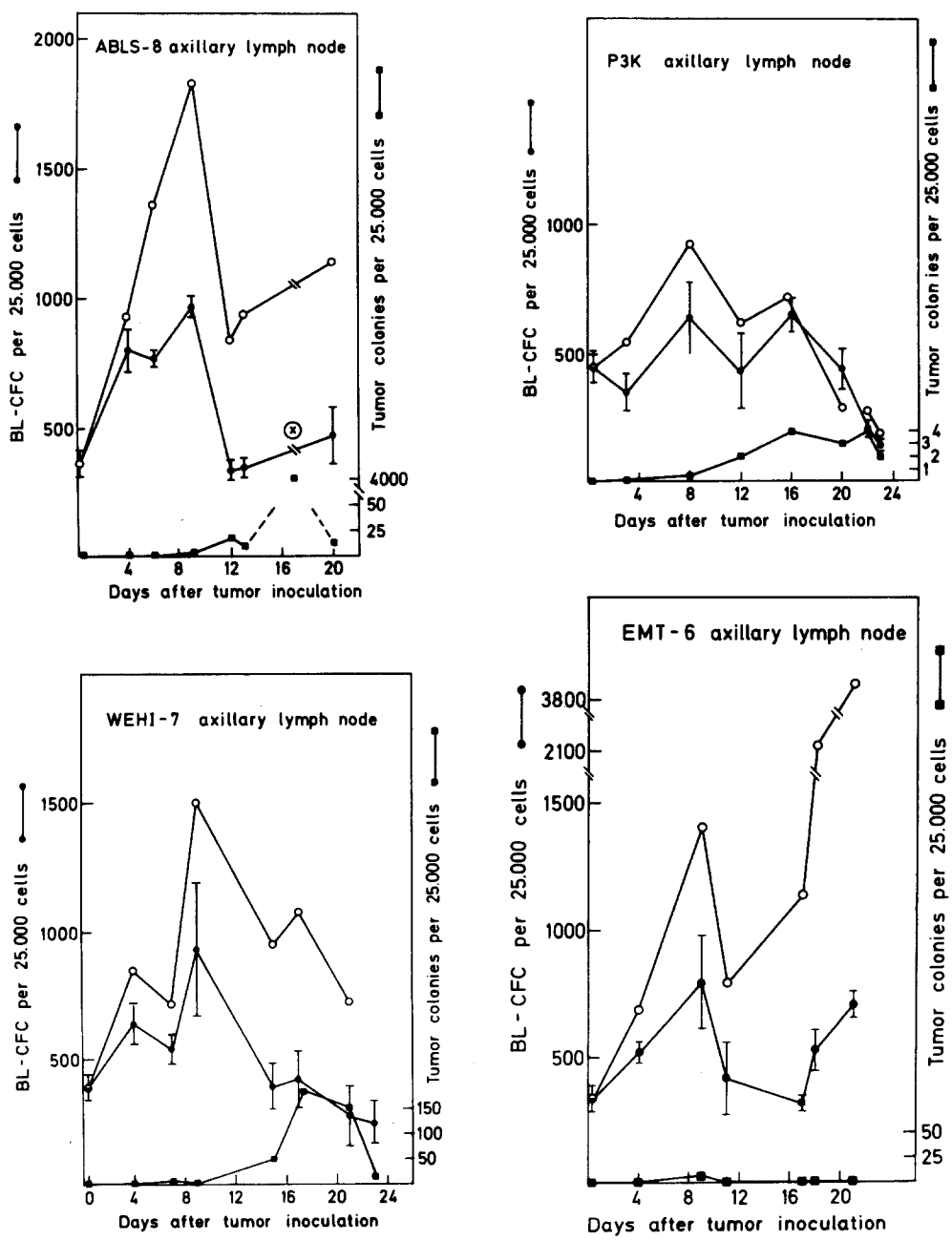

Fig. 3.

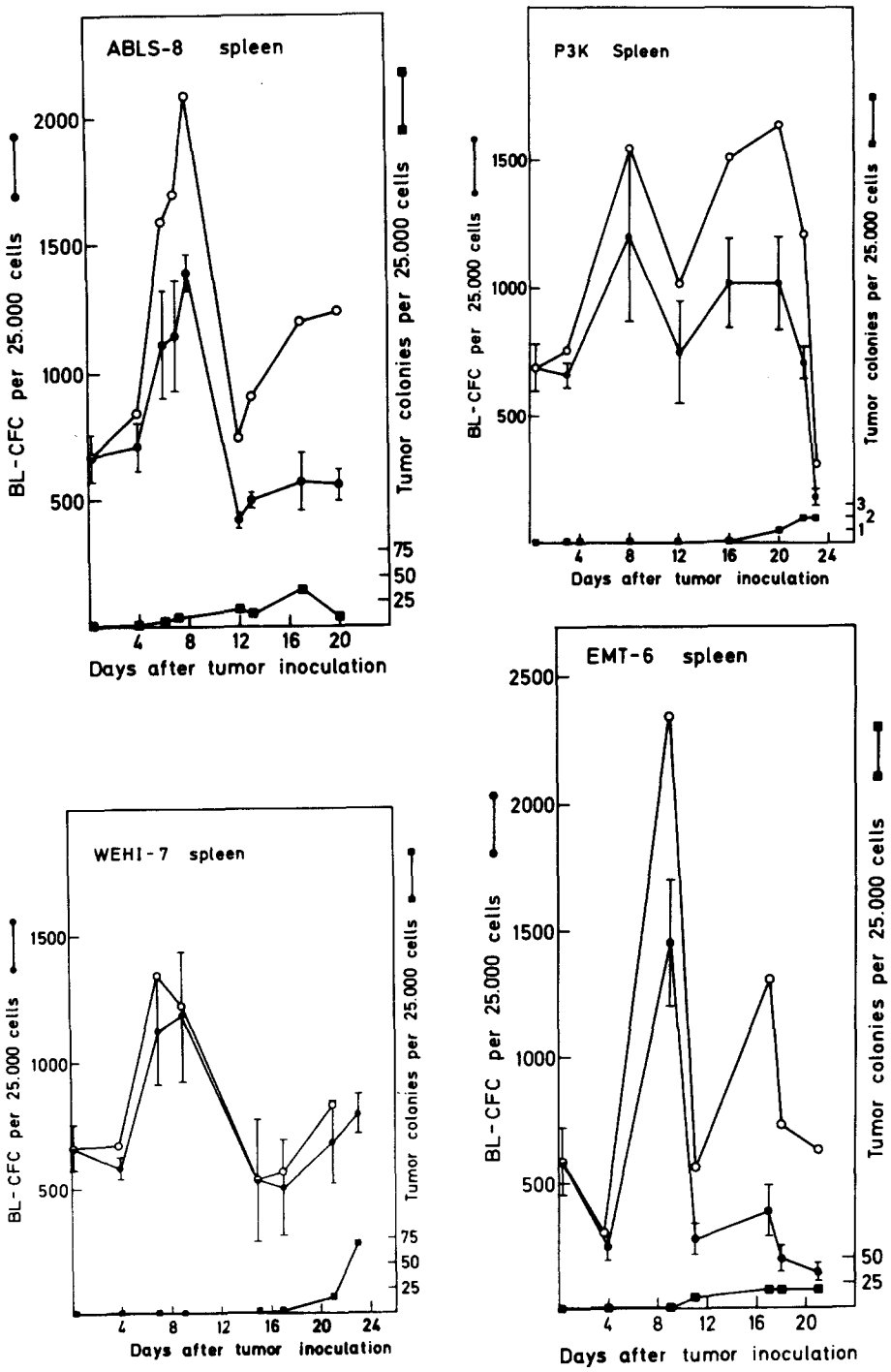

Fig. 4

Figs. 3 *and* 4. *Frequency of BL-CFC in axillary lymph nodes and spleen of tumor bearing mice. The data represent the corrected mean values of groups of* 3 *animals (conf. Material and Methods). Colonies were scored in four replicate culture dishes and S.D. calculated (vertical bars). The open circles reflect the increase in total BL-CFC numbers as a result of lymphoid organ weight increase and have been calculated as:*

$$\frac{\textit{BL-CFC frequency in a tumor bearing group} \times \textit{organ weight}}{\textit{mean organ weight in the control groups}}$$

by combining the fitted weight data in Fig. 2 *and the values for BL-CFC frequency in the present Figs.* ⊗: *These cultures were crowded with tumor colonies and BL-CFC numbers could not be counted.*

*Table 1. Bone marrow cell numbers in mice bearing transplantable tumors**

Days after tumor inoculation	ABLS-8 $\times 10^6$/ml	P3K $\times 10^6$/ml	WEHI-7 $\times 10^6$/ml	EMT-6 $\times 10^6$/ml	Control $\times 10^6$/ml
3–4†	11.9	11.7	12.8	12.3	16.8
7–9†	10.2	11.0	13.0	12.5	11.9
14	11.8	12.0	10.0	11.8	12.3
17	4.7	14.2	7.8	22.5	23.7
22		15.0			18.3

*Data represent numbers of viable bone marrow cells obtained from 6 femora in 5 ml EBSS.
†Data are means of two separate experiments.

Table 2. Effect on colony growth of serum obtained from late tumor bearing mice. Number of tumor colonies developed after incubation with serum from tumor bearing mice

Tumor strain	P3K-serum			ABLS-8 serum			WEHI-7 serum			Control			Saline
	5%	2½%	0.6%	5%	2½%	0.6%	5%	2½%	0.6%	5%	2½%	0.6%	
P3K‡	240	265	290	275	345	320	3	50	105	0	100	100	185
ABLS-8§	380	450	470	15	570	505	0	255	410	0	30	435	415
WEHI-7§	550	525	495	150	565	645	25	465	500	0	375	590	520
EAT-6§	135	155	140	150	130	160	10	210	135	25	160	170	110

‡2500 cells/dish. Data represent the mean colony counts of 2 plates.
§1000 cells/dish.

bearing ABLS-8 and WEHI-7 tumors during the last stages of tumor growth.

GM-CFC in spleen and BM

Figure 5 shows the incidence of GM-colonies in the spleens and BM of tumor bearing mice. Growth of the WEHI-7 tumor did not alter GM-CFC frequency, whereas the bone marrow of ABLS-8 and EMT-6 tumor bearing animals and the spleens of ABLS-8, P3K and EMT-6 tumor bearing mice showed a 5–200 fold increase in GM-colony frequency. At all times after tumor innoculation, no changes were observed in the proportions of neutrophil, mixed neutrophil–macrophage or macrophage colonies, when compared with normal control mice. No tumor colonies were detected in the bone marrows of ABLS-8, P3K or EMT-6 tumor bearing mice. Low numbers of tumor cells were detected however, in the bone marrows of WEHI-7 tumor bearing animals.

Loss of serum inhibition of tumor colony growth

Normal mouse serum was found to exert a strong inhibitory effect on the growth of tumor colonies at concentrations from 2½ to 5% (Table 2). Serum obtained from tumor bearing mice during the first two weeks after inocculation did not differ from normal serum with respect to this inhibitory effect. In contrast, data in Table 2 show that serum obtained from some of the groups of mice during the late period of tumor growth (the end of the third week) had partially lost the inhibitory property when tested against the four tumor types. Only serum obtained from WEHI-7 bearing mice showed inhibition in the tumor colony assay comparable to that of control serum. It is evident from Table 2 that this loss of serum inhibition was completely unspecific.

Mixing experiments

From 100 to 2500 tumor cells (ABLS-8, P3K and WEHI-7) were mixed with 10.000 normal spleen cells prior to assaying for B lymphocyte colony formation. No stimulatory or inhibitory effect on B lymphocyte growth was observed by this co-cultivation of tumor cells and normal lymphoid cells. Similarly mixing experiments with WEHI-7 tumor cells (1000–4000) and normal bone marrow cells did not inhibit or potentiate the formation of GM-colonies.

DISCUSSION

The present data show that increased levels of BL-CFC occur in the regional lymph nodes and spleens and that GM-CFC levels increase in the bone marrow and spleens of mice bearing

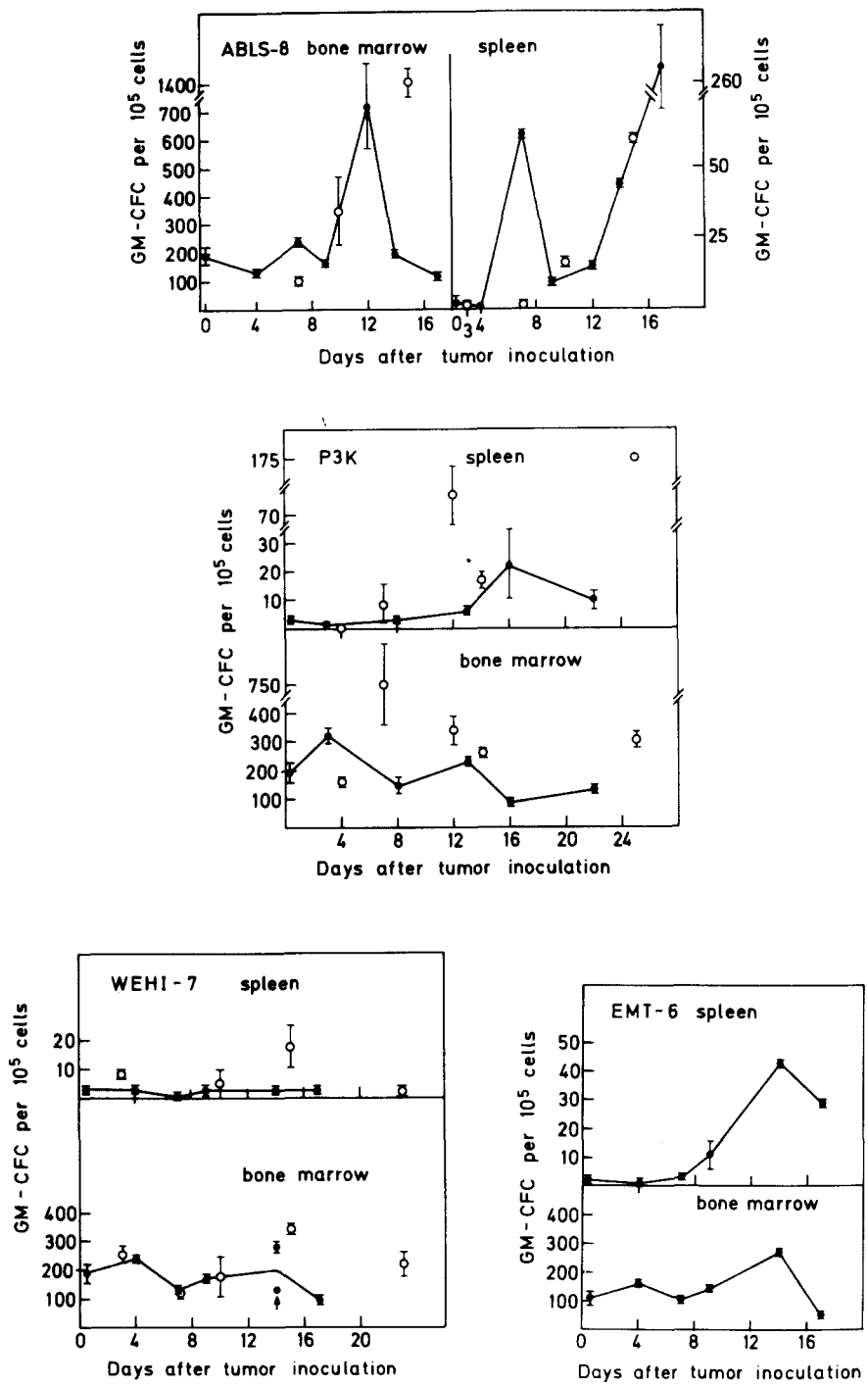

Fig. 5. Frequency of GM-CFC in spleen and bone marrow of tumor bearing mice. Data are mean values of pools of 3 animals cultured in four replicate culture dishes. Open circles represent data from single animals. Vertical bars represent ± S.D. All data are corrected mean values (cf. Material and Methods).
↑: Tumor colonies present in the bone marrow culture.

subcutaneously transplanted B lymphoma, plasmacell tumor, or a mammary carcinoma. Mice with transplanted T lymphoma showed only elevated numbers of BL-CFC whereas the GM-CFC levels were not influenced by tumor growth. Lymphoid organ weights and numbers of bone marrow cells were only slightly affected by tumor growth in mice transplanted with B and T lymphomas whereas the mammary carcinoma-bearing mice showed an enormous enlargement of both the regional lymph nodes and spleen. The observed increase in lymphoid organ weights or bone marrow cell counts in tumor bearing mice was not due to infiltration of the organs with tumor cells since no or very few tumor cells could be detected in the respective organs.

BL-CFC represent a subpopulation of B

lymphocytes with a potential for cell proliferation *in vitro* i.e. colony formation [4]. *In vivo* this subpopulation represents 5–10% of all B lymphocytes, from immature, early B lymphocytes to the postantigen, memory type B lymphocyte. The function of the colony-forming cells and factors which regulate the numbers of BL-CFC *in vivo*, are poorly understood [13, 14].

During the early period of tumor growth, both the incidence and the total numbers of BL-CFC were elevated in the regional lymph nodes and the spleen irrespective of the tumor type employed. The mesenteric lymph nodes seemed to be unaffected by tumor growth. The data may suggest that excessive proliferation of neoplastic B lymphocytes (i.e. ABLS-8 and P3K tumors) does not specifically influence normal B lymphocyte colony forming cell levels in tumor-bearing mice. The increased levels of BL-CFC may simply reflect a host response to the presence of antigens introduced by tumor proliferation (e.g. dead tumor cells or secondary infections). Also specific antigens, present on the tumor cell types studied might trigger B lymphocytes *in vivo* and lead to increased levels of BL-CFC. Since methods are not available to test B lymphocyte colony cells for antibody production against such tumor associated antigens, the mechanisms underlying the observed response can not be fully explored. The negative results of the tumor-spleen cell mixing experiments, indicate however that the tumor cells themselves do not produce factors which influence B lymphocyte colony formation.

During the latter part of the tumor-bearing period the BL-CFC response became more complex. The lymphoma and plasmacytoma-bearing mice all showed normal or decreased incidences of BL-CFC, whereas the mice transplanted with mammary carcinoma cells showed increased levels of colony forming cells in the regional lymph nodes. Due to the increase in lymphoid organ weight in the late part of the experimental period, the total number of BL-CFC in ABLS-8 and WEHI-7 bearing animals increased, whereas plasmacytoma-bearing mice showed reduced frequency and numbers of BL-CFC in the regional lymph nodes and the spleen. A possible explanation for the tendency towards decreased BL-CFC levels may be that these stressed animals exhibit increased amounts of circulating corticosteroids; hormones which *in vivo* selectively depress BL-CFC relative to non-colony-forming lymphocytes [9, 13].

It has been shown that bone marrow and spleen GM-colony levels are elevated in mice transplanted with syngeneic plasmacell or breast tumors [15]. Similarly, granulocytopoiesis and the number of spleen colony-forming cells are increased in some tumor-bearing mice [1, 2]. Thus, the present observations showing a 5–200 fold increase in GM-colony forming cells in the spleen of B lymphoma, plasmacytoma and mammary tumor bearing mice, confirm and extend these earlier observations. The observed increase might reflect an unspecific protective response against the growing tumor cells which may be mediated by increased levels of circulating GM-CSF known to occur in tumor-bearing mice [15]. Surprisingly, no effect on GM-colony forming cell levels was observed in T lymphoma-bearing mice, where in fact the frequency of bone marrow derived GM-CFC tended to decline during the late tumor-bearing period. This obvious lack of a "protective response" in WEHI-7 bearing mice might bear some relationship to the more frequently observed infiltration of the host lymphoid organs, spleen and bone marrow by this tumor type. Differences in levels of GM-colonies in tumor-bearing mice might moreover reflect different capabilities of individual tumors to produce *in vivo* the factors which stimulate colony formation *in vitro* [15–16].

Normal unfractionated mouse serum exhibits a high, non-specific, colony-inhibitory activity when added to cultures of B lymphocytes, GM-precursors, lymphoid leucaemic, plasmacytoma and mastocytome cells [17]. These findings were confirmed in the present study where non-specific inhibitory activity was present in serum from early lymphoma and plasmacytoma bearing mice. In contrast, serum obtained from late B lymphoma- and plasmacytoma-bearing mice did not inhibit colony formation *in vitro* by any of the four cell types used. However, serum from late T lymphoma-bearing animals showed normal inhibition of colony formation. It is known that serum inhibitory activity against GM-CFC is lowered by starvation of the serum donor [18] and starvation due to the weak condition of tumor-bearing animals might be held responsible for the observed lack of inhibitory activity in serum of these mice. Factors occurring in serum of late tumor-bearing mice might, on the other hand, neutralize its inherent inhibitory activity or perhaps directly stimulate growth of tumor cells leading to the observed phenomenon.

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Prospective Study of the α and β Subunits of Human Chorionic Gonadotrophin in the Blood of Patients with Various Benign and Malignant Conditions*

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Abstract—*A prospective study of HCG-β (the human chorionic gonadotrophin and its free β subunit) and of H-α (the free α subunit) was carried out in the plasma of 414 patients with malignant tumours and 99 patients with benign diseases (chiefly benign tumours and benign conditions associated with tissue hyperplasia).*

HCG-β and excessive free α subunit concentrations were found with a similar frequency (in 16–21% of the patients); both were found as frequently in the benign and in the malignant conditions; furthermore there was no clearcut predisposition according to the pathology except for choriocarcinomas.

However the benign and the malignant conditions differed from each other in that the concentrations of HCG-β were significantly higher in the latter whereas the very high plasma concentrations (>50 ng/ml) of HCG-β or of H-α were specific for malignant conditions.

The patients with HCG-β were not the same as those with excessive H-α concentrations: 75% of the latter had no detectable HCG-β, indicating that the production of the α subunit can be regulated independently from that of HCG-β.

Unlike in the patients with excessive H-α, in the patients with HCG-β there was a very significant correlation between the HCG-β and the corresponding free α subunit concentrations: this indicates that the production of HCG-β was linked to that of the free α subunit whereas the reverse was not necessarily true.

The concentrations of HCG-β in plasma were qualified according to the elution profiles on Sephadex chromatography and the relative affinity of our antibody to HCG and the free β subunit. The presence of the free β subunit and of abnormal chorionic gonadotrophin was demonstrated.

INTRODUCTION

HUMAN chorionic gonadotrophin (HCG) and its free α subunit (HCG-α) are normally secreted by the placenta [1].

The α subunit of HCG is practically identical to that of the follicle-stimulating hormone (FSH), the luteinizing hormone (LH) or the thyroid-stimulating hormone (TSH) and cannot be distinguished by radioimmunoassay. Therefore, this common free α subunit (H-α) is found in the blood of normal, non-pregnant individuals. Its concentration in blood increases when the secretions of FSH, LH or TSH are stimulated, as in postmenopausal women or in patients with primary hypothyroidism [2]. However, once the normal limits of the free H-α concentrations have been determined, men or non-pregnant women with normal pituitary function but excessive free H-α concentrations should be suspected of ectopic secretion.

HCG, on the other hand, is immunologically unique because of its characteristic β subunit (HCG-β). It was hoped therefore that HCG-β would be a specific tumour marker in non-pregnant individuals [3, 4]. However, as HCG was found recently in normal testes [5], in normal pituitaries [6] and in the blood of

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patients with gastro-intestinal inflammatory disorders [1], this view must be reconsidered.

The present work constitutes a prospective study of H-α (as the common free α subunit) and of HCG-β (as the free β subunit and as part of the molecule of HCG) in the blood of more than 500 patients admitted consecutively in our hospital. The concentrations of these proteins in blood were correlated with the benignity or the malignancy of the disease and with the pathological diagnosis; they were also correlated with each other to help in the understanding of their genetic regulation.

MATERIAL AND METHODS

1. *Design of the study*

HCG-β (as the free β subunit and as part of the molecule of HCG) and H-α (as the free α subunit, common to the various glycoproteic hormones including HCG) were measured in a prospective fashion, prior to therapy, in all the patients hospitalized in our Institute. The plasma concentrations of these proteins were correlated with the pathology; they were also correlated with each other. The normal controls were members of the medical staff and healthy people consulting for a preventive medical examination.

2. *Patients* (*see also Tables* 1 *and* 2)

(a) *Malignant tumours:* 414 *patients.* There were 116 mammary carcinomas, 50 bronchial carcinomas (including 24 epidermoid carcinomas, 14 oat-cell carcinomas, 4 undifferentiated large cell carcinomas), 37 gastrointestinal tumours (including 14 gastric, 6 colic, 11 rectal and 2 pancreatic carcinomas), 45 gynaecologic carcinomas (29 of the cervix, 10 of the endometrium and 6 of the ovaries), 12 thyroid carcinomas (8 follicular, 3 medullary and 1 undifferentiated carcinoma), 20 melanomas, 5 testicular tumours (including 3 choriocarcinomas), 29 lymphomas or leukaemias (14 lymphosarcomas, 4 Hodgkin's diseases, 8 chronic lymphoid leukemias and 3 myelomas), 51 carcinomas of the oropharynx or larynx (including 13 of the larynx, 10 of the tongue and 6 of the gums), 38 miscellaneous (including 13 basal cell carcinomas of the skin, 9 sarcomas, 2 bladder carcinomas, 6 cerebral tumours and 2 hypernephromas).

Table 1. *Patients with significant HCG-β concentrations*

Diagnosis	Numbers	Percentages	Ranges of concentrations (ng/ml)	Median concentrations (ng/ml)
Malignant tumours (68/414 = 16%)				
Mammary carcinomas	24/116	21%	1.6–7.6	2.4
Bronchial carcinomas (5 squamous cell, 1 oat cell carcinomas)	6/50	12%	2.1–3.5	2.5
Gastro-intestinal carcinomas (1 parotid, 1 gastric, 1 gallbladder, 1 pancreatic, 3 rectocolic carcinomas)	7/37	19%	1.6–17	1.7
Gynaecologic carcinomas (4 cervical, 2 endometrial, 1 ovarian carcinomas)	7/45	16%	2.4–>50	2.9
Thyroid carcinomas (2/8 follicular, 1/3 medullary carcinomas)	3/12	25%	1.6–2.8	1.8
Melanomas	3/20	15%	1.7–>50	1.8
Choriocarcinomas	3/3	100%	>50 in all	>50
Lymphomas and leukemias	3/29	10%	1.6–2.3	1.7
Basal cell carcinomas of skin	2/13	15%	1.6–2.8	—
Carcinomas of the oropharynx and larynx	3/51	6%	1.6–4.4	2.3
Miscellaneous (including 1 mesothelioma, 2 hypernephromas, 1 bladder and 1 bladder + oat cell carcinomas)	7/38	18%	1.7—>50	2.4
Benign diseases (16/99 = 16%)				
5 multinodular goiters, 1 parathyroid adenoma, 1 uterine fibroma, 1 benign ovarian cysts, 2 chronic adenitis, 1 severe osteoporosis, 1 liver + bone tuberculosis, 1 liver cirrhosis, 2 perforated colic diverticulitis, 1 severe int. hemorroids			1.6–2.6	1.75

Table 2. Patients with excessive free α subunit concentrations

Diagnosis	Numbers	Percentages	Ranges of concentrations (ng/ml)	Median concentrations (ng/ml)
Malignant tumours (87/414 = 21%)				
Mammary carcinomas	35/116	30%	2.5–>50	7
Bronchial carcinomas (4 squamous cell, 1 oat cell carcinomas)	5/50	10%	2.5–12	3.3
Gastro-intestinal carcinomas (3 gastric, 3 rectal, 2 pancreatic carcinomas)	8/37	22%	3.4–13	8.2
Gynaecologic carcinomas (12 cervical, 1 endometrial, 3 ovarian carcinomas)	16/45	36%	2.6–>50	3.7
Thyroid carcinomas (3/9 follicular carcinomas)	3/12	25%	4.5–5	5
Melanomas	6/20	30%	2.7–30	5
Choriocarcinomas	2/3	67%	7.8–>50	—
Lymphomas and leukemias (Hodgkin's disease)	1/29	3%	>50	—
Basal cell carcinoma of skin	1/13	8%	3	—
Carcinomas of the oropharynx and larynx	6/51	12%	2.5–7.6	3.4
Misellaneous (2 sarcomas, 1 mesothelioma, 1 prostatic carcinoma)	4/38	11%	3.2–12	8.5
Benign diseases (16/96 = 17%)				
9 multinodular goiters, 1 uterine fibroma + ovarian cysts, 1 endometrial hyperplasia, 1 fibrocystic hyperplasia of breasts, 1 liver + bone tuberculosis, 2 perforated colic diverticulitis, 1 essential hypertension			2.5–8.2	3.9

(b) *Benign diseases: 99 patients.* There were 34 euthyroid nodular goiters, 4 parathyroid adenomas, 4 diseases of the breast (3 fibrocystic hyperplasias, 1 intraductal papilloma), 16 gynaecologic diseases (including 3 ovarian cysts, 7 uterine fibromas, 2 endometrial hyperplasias, 1 polyp of the cervix), 10 diseases of the liver and the gastrointestinal tract (including 1 liver cirrhosis, 1 liver steatosis, 1 chronic cholecystitis, 1 liver tuberculosis, 2 perforated colic diverticulitis, 1 duodenal ulcer), 31 miscellaneous (including 3 benign chronic adenitis, 2 prostatic adenomas and 1 retroperitoneal fibrosis).

3. *Radioimmunoassays*

All assays were carried out in duplicate by the double-antibody technique. Highly purified HCG-β subunit (SB-6) and the corresponding rabbit antibody were generously provided by the National Institute of Arthritis, Metabolic and Digestive Diseases (N.I.H., Bethesda, Md). Highly purified LH-α subunit (~100 i.u. IRP-HMG per mg) and the corresponding rabbit antibody were obtained from commercial sources (Calbiochem, U.S.A.). HCG-β was iodinated by the method of Vaitukaitis *et al.* [7]; H-α was iodinated by the chloramine T method [8]. The first incubation was carried out at 4°C during 3–5 days; the 0.4 ml incubates were composed as follows: 0.1 ml of plasma, 0.1 ml of phosphate buffer containing 2.5% rabbit serum, 0.1 ml of phosphate buffer with 0.5% bovine serum albumin containing ~10,000 counts/min of labeled hormone, 0.1 ml of the same buffer containing the antibody (final dilution of the antibody to HCG-β: 1/120,000 and of the antibody to H-α: 1/4,000). At the end of the first incubation an appropriate amount of sheep anti-rabbit gamma globulin was added and the second incubation was carried out at 4°C during 6 hr. The samples were then centrifuged, the supernatants decanted and the precipitates counted. In the absence of unlabeled ligand, 30–40% of the labeled ligand was precipitated by the second antibody (B_0).

Remarks about the HCG-β radioimmunoassay: the HCG-β standards were dissolved in normal human serum; however some material reacting like HCG-β was still found in the plasma of many normal controls; the highest plasma concentration of this material in the normal controls was 1.5 ng/ml (see Results) and B/B_0 was then 60%. The specificity of the HCG-

β assay was investigated: there was a cross-reaction of the order of 12% with HCG but no cross-reaction with LH-β, LH, FSH and TSH; the assay measured therefore the free HCG-β subunit and, with much less sensitivity, HCG itself (see Discussion).

Remarks about the H-α radioimmunoassay: the sensitivity of the assay was 0.2 ng/ml; it was specific for the free α subunit since the cross-reaction with HCG or LH repurified by column chromatography was less than 5%.

In view of the structural similarities of HCG with LH, plasma LH was measured in the normal controls to find out if the material cross-reacting with HCG-β in our radioimmunoassay correlated with plasma LH. The luteinizing hormone (1 mg = 66 mg ± 6 LER 907) and the antibody used in this assay were obtained from the Radiochemical Centre of Belgium (I.R.E.); the assay was similar to that of H-α.

4. *Column chromatography*

The serum of some patients with very high HCG-β concentrations was fractionated on Sephadex G-100 in order to determine the relative importance of HCG and of its free β-subunit [9]. The void volume (V_o) of the column, measured by the elution of dextran blue, was 55 ml; its total volume (V_T) was 150 ml. The column was calibrated by determining the elution peaks (V_e) of labeled HCG, β and α subunits. In separate experiments, standards of unlabeled HCG, β and α subunits were similarly eluted: their respective V_e/V_o: 1.31, 1.38 and 1.75 were identical to those of the labeled proteins; the corresponding kav were: 0.20, 0.25 and 0.48. The concentrations of the β and α subunits were measured as described above in 0.1 ml of each elution fraction. The concentrations of HCG were measured apart with an antibody to HCG which could not be used in unfractionated plasma because of its cross-reaction (18%) with LH; HCG used in this assay was obtained from the National Institute of Health (U.S.A.) and the antibody was obtained from the I.R.E. (Belgium); the assay was similar to that of H-α.

5. *Statistical analysis*

The data were evaluated by a non-parametric analysis of variance [10].

RESULTS

1. *Plasma concentrations in the normal controls*

(a) "*HCG-β*". A material cross-reacting with HCG-β was found in the normal controls. The mean concentrations and the standard deviations (s.d.) were as follows: 0.34 ng/ml (s.d. 0.52) in 26 men, 0.47 ng/ml (s.d. 0.51) in 10 premenopausal women and 0.58 ng/ml (s.d. 0.44) in 16 postmenopausal women. There was no significant difference between the three populations ($P=0.31$); therefore all the data were grouped and the highest "normal concentration", i.e. 1.5 ng/ml, was taken as the "normal upper limit".

(b) "H-α". The mean H-α concentrations were as follows: 0.53 ng/ml (s.d. 0.43) in the 26 men, 0.36 ng/ml (s.d. 0.47) in the 10 premenopausal women and 1.39 ng/ml (s.d. 0.51) in the 16 postmenopausal women. The concentrations in the postmenopausal women were significantly higher ($P<0.0001$) than in the two other groups; therefore, the highest concentration found in the postmenopausal women, i.e. 2.4 ng/ml was taken as the normal upper limit.

(c) *Correlation analysis between "HCG-β" and H-α concentrations.* There was no significant correlation between the plasma concentrations of H-α and those of the material cross-reacting with HCG-β.

(d) *LH.* With regard to LH, the following mean concentrations were found: 2.6 ng/ml (s.d. 1.3) in the men, 4.7 ng/ml (s.d. 5.5) in the premenopausal women and 11.8 ng/ml (s.d. 3.4) in the postmenopausal women; there was no significant correlation between the concentrations of LH and those of the material cross-reacting with HCG-β ($P=0.13$) but the concentrations of LH correlated very significantly with those of H-α ($P<0.0001$).

2. *HCG-β in the patients: plasma concentrations above* 1.5 ng/ml (*Table* 1)

(a) *Frequency of these HCG-β concentrations according to the disease.* Malignant tumours vs benign diseases: 68 of the 414 patients with malignant tumours (16%) and 16 of the 99 patients with benign diseases had high plasma concentrations of HCG-β, thus the same percentage in each population.

According to the pathology of the malignant tumours, except for the choriocarcinomas which obviously formed a distinct entity, the frequency of high HCG-β concentrations was of the same order of magnitude in all types of malignant tumours although the frequency might have been somewhat lower in the tumours of the oropharynx and larynx.

According to the pathology of the benign diseases, it is of interest that 5 of the 34 benign nodular goiters (15%) had high HCG-β concentrations in plasma.

(b) *Range of these plasma concentrations according to the disease.* HCG-β measured by our radioimmunoassay was actually the free β subunit, the whole molecule of HCG or a combination of both. With these reservations in mind (see Discussion), it is interesting to see that the plasma concentrations of HCG-β were significantly higher in the patients with malignant tumours. Indeed, of the 68 patients with a malignant tumour described in Table 1, 7 had concentrations above 50 ng/ml (3 had a choriocarcinoma, 1 an oat-cell carcinoma plus a carcinoma of the bladder, 1 a mesothelioma, 1 a melanoma and 1 a carcinoma of the endometrium) whereas the highest concentration in the benign diseases was 2.6 ng/ml; furthermore, in the remaining 61 patients with a malignant tumour, the plasma concentrations of HCG-β (mean = 2.8, median: 2.3, range: 1.6–17 ng/ml) were still significantly higher ($P = 0.004$) than in the patients with benign diseases (mean = 1.9, median: 1.8, range: 1.6–2.6 ng/ml).

However, among the patients with malignant tumours there was no significant difference of concentration according to the pathology of the tumour except for the choriocarcinomas where the plasma concentrations were always > 50 ng/ml.

(c) *Correlation analysis between these HCG-β and the H-α concentrations.* The correlation between these HCG-β concentrations (Table 1) and the corresponding H-α concentrations was statistically very significant ($P \sim 0.001$).

3. *The free α subunit in the patients: plasma concentrations above* 2.4 ng/ml (*Table* 2)

(a) *Frequency of these high H-α concentrations according to the disease.* Malignant tumours vs benign diseases: 87 of the 414 patients with malignant tumours (21%) and 16 of the 95 euthyroid patients with benign diseases (17%) had excessive H-α concentrations; thus, again, the percentage was similar in the benign and in the malignant conditions.

According to the pathology of the malignant tumours: the frequency was perhaps somewhat higher in the patients with mammary carcinomas, gynaecologic carcinomas or melanomas and to a lesser extent with thyroid or gastrointestinal carcinomas; it is of interest that only one of the three patients with a choriocarcinoma had very high (> 50 ng/ml) H-α concentrations in plasma.

According to the pathology of benign diseases: it is remarkable that 9 of the 34 euthyroid nodular goiters (26%) had high H-α concentrations in plasma.

(b) *Range of these plasma concentrations according to the disease.* As shown in Table 2, the highest plasma concentration of H-α in the 16 patients with benign diseases was 8.2 ng/ml whereas 27 of the 87 patients with a malignant tumour had concentrations above 8.2 ng/ml; 5 patients with a malignant tumour had actually plasma concentrations above 50 ng/ml (1 choriocarcinoma, 2 generalized mammary carcinomas, 1 ovarian carcinoma, 1 Hodgkin's disease); although the medians were similar (respectively 3.9 and 4.5 ng/ml) and the difference was not statistically significant between the benign and the malignant conditions it is obvious that the patients with plasma concentrations above 50 ng/ml belonged to a distinct population: they all had a malignant tumour.

Again, as for HCG-β, there was no difference of H-α concentration according to the pathology of the malignant tumour.

(c) *Correlation analysis between these H-α and the HCG-β concentrations.* No significant correlation was found between these H-α and the corresponding HCG-β concentrations; actually, among the 103 patients described in Table 2, 77 had no detectable HCG-β in their plasma; furthermore, among the 5 patients with plasma H-α concentrations in excess of 50 ng/ml, 3 had no detectable HCG-β.

4. *Chromatographic characterization of HCG and its subunits*

The elution profiles were variable. Four examples are presented in Figs. 1–4. Figure 1: patient De Pot (oat cell carcinoma and carcinoma of the bladder): HCG, the free β and the free α subunits were all found at high concentrations in the serum of this patient; however, the elution profiles of these proteins were normal (respective kav: 0.18, 0.25 and 0.44). Figure 2: patient Hou (melanoma): the serum contained essentially HCG and its free β subunit (normal kav again: respectively 0.19 and 0.25). Figure 3: patient Ar (very malignant choriocarcinoma): only HCG was found in the serum (normal kav again: 0.18). Figure 4: patient Del (carcinoma of the endometrium): although fresh serum was passed on column chromatography, only a very abnormal HCG was found (very low kav: 0.09 and thus presumably increased molecular weight).

DISCUSSION

With regard to HCG-β, our antibody was specific but was more sensitive to the free β subunit than to the chorionic gonadotrophic hormone itself. Therefore our measurements in

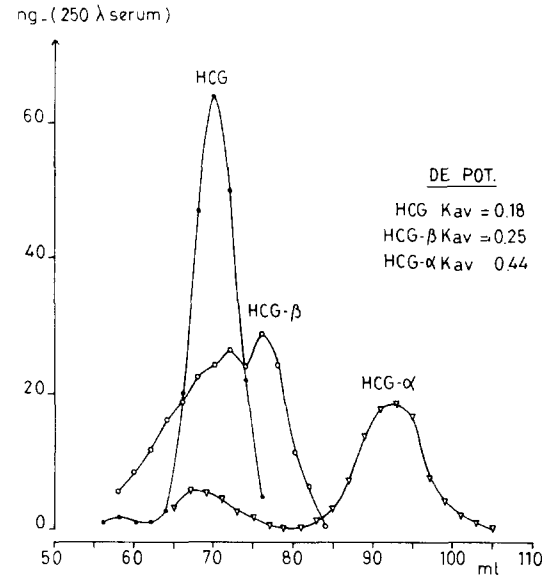

Fig. 1. Elution profiles of the ectopic proteins on Sephadex G-100. Patient De Pot.: oat cell carcinoma and carcinoma of the bladder.

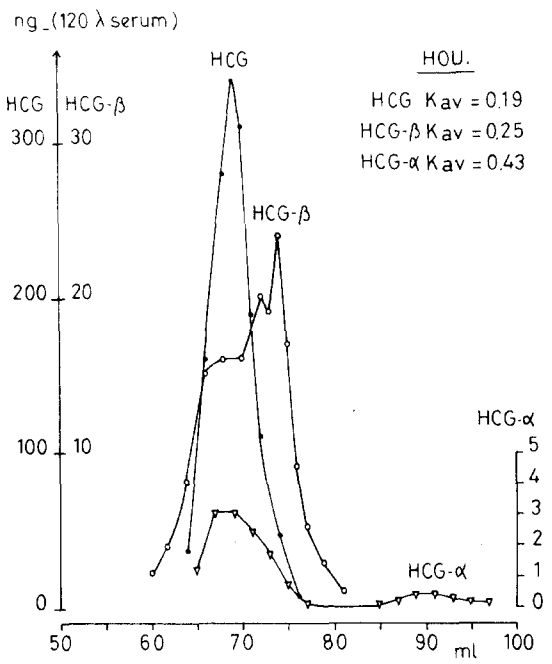

Fig. 2. Elution profiles of the ectopic proteins on Sephadex G-100. Patient HOU: melanoma.

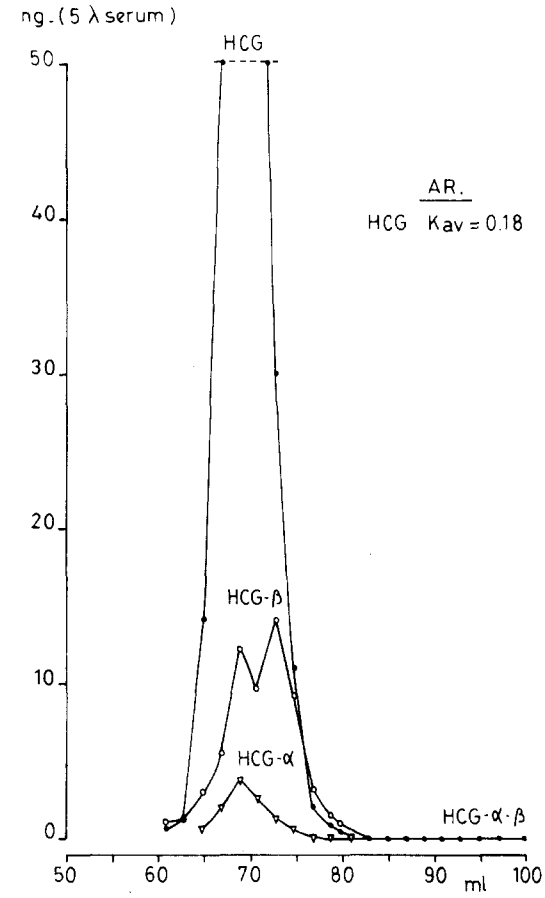

Fig. 3. Elution profiles of the ectopic proteins on Sephadex G-100. Patient Ar.: very malignant choriocarcinoma.

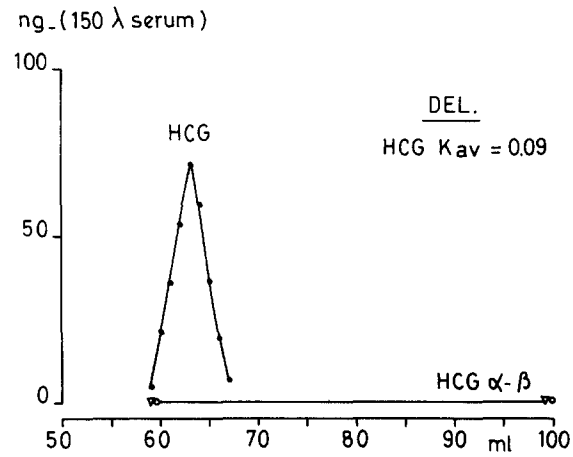

Fig. 4. Elution profiles of the ectopic proteins on Sephadex G-100. Patient Del.: carcinoma of endometrium.

the patients were not really quantitative: the plasma concentrations of HCG-β corresponded to relatively small concentrations of the free β subunit, to relatively high concentrations of HCG, or to a combination of both. This was illustrated by our chromatographic analyses. The latter showed in addition that plasma HCG (or its free β subunit) had sometimes an abnormal structure with increased molecular weight, as found in pre-hormones: such abnormalities could decrease the affinity of the antibody to the β subunit and further interfere with the quantitative evaluation. Finally, part of what was measured in plasma was presumably a non-specific material, cross-reacting with the antibody to HCG-β, since present in many normal individuals. The lower affinity of our antibody to normal or to abnormal HCG and

the presence in the plasma of a non-specific background could mask differences of HCG concentrations between the various populations of patients and mask also quantitative correlations with the α subunit. When such differences and correlations were found they were thus all the more significant but had to be qualified, particularly in terms of preferential free β subunit production, as will be discussed below.

Our data confirmed at first the work of Vaitukaitis *et al.* [1] who showed that HCG-β in the blood of non-pregnant individuals was not specific for malignant tumours. However, our finding that significant plasma concentrations of HCG-β were as frequent in the patients with benign diseases as in the patients with malignant tumours was unexpected. It is of interest, therefore, that the patients admitted at our Institute with benign diseases were certainly selected: most of them had benign tumours such as nodular goiters, parathyroid adenomas, uterine fibromas, papillomas of the breast or had benign conditions associated with tissue hyperplasia such as hyperplasia of the endometrium, fibrocystic hyperplasia of the breast, ovarian cysts, inflammations of the gastrointestinal tract, chronic adenitis. Another unexpected finding was the absence of any predisposition according to the pathology, both in the benign and in the malignant conditions, except for the choriocarcinomas; Braunstein *et al.* [3], in the first extensive study of HCG-β in patients with malignant tumours found a higher incidence of HCG-β in the patients with gastrointestinal carcinomas, although their study was not prospective. This lack of specificity suggests, as proposed by Vaitukaitis *et al.* [1], that a common anomaly, perhaps enhanced cellular proliferation, was the source in all instances of plasma HCG-β. This lack of specificity indicates also that the measurement of HCG-β in plasma has not much interest as a screening procedure, except probably when very high plasma concentrations (above 50 ng/ml) are found. Indeed, the benign and the malignant conditions differed in that the plasma concentrations of HCG-β were significantly higher in the patients with a malignant tumour. Since our antibody to HCG-β was much more sensitive to the free β subunit than to HCG itself, this difference of concentrations could result to some extent from a relatively higher production of the free β subunit in the malignant conditions; this difference could then be both qualitative and quantitative; in this regard, it may be relevant that Vaitukaitis *et al.* [2] showed that the free β subunit was an index of more severe malignancy in the patients with trophoblastic tumours [1].

With regard to the free α subunit, the percentage of patients with abnormal plasma concentrations was similar to that found for HCG-β. This percentage, again, was practically the same in the benign and in the malignant conditions, and there was no clearcut predisposition according to the pathology except probably for the choriocarcinomas, although it is remarkable that unlike for HCG-β not all the patients with choriocarcinomas had elevated plasma concentrations of HCG-α. Furthermore, as for HCG-β, the very high plasma concentrations of the α subunit (above 50 ng/ml) were found exclusively in the malignant conditions. Consequently, the measurement of the free α subunit in plasma is of no more interest than that of HCG-β as a screening procedure and a common cellular anomaly might be responsible for both excessive H-α and HCG-β productions. It should be noted however that the patients with excessive free α subunit in plasma were not the same as those who had HCG-β: indeed, there was no correlation between their free α subunit concentrations and their corresponding HCG-β concentrations; furthermore, most of these patients, even with the highest H-α concentrations, had no detectable HCG-β. This indicates that the production of H-α can be regulated independently from that of HCG-β, H-α being secreted as an isolated subunit or in association with the other glycoproteic hormones.

Although the patients with high free α subunit concentrations were not the same as those with HCG-β, it is interesting that in the latter we found a very significant correlation between the HCG-β and the corresponding free α subunit concentrations. This is comparable to what is found during pregnancy, since the normal placenta secretes the free α subunit together with HCG [4]; to some extent, this is also comparable to what is found in the normal postmenopausal women and in primary hypothyroidism where the free α subunit secretion increases in parallel with that respectively of FSH and TSH [2]; finally, this is similar to our own observations in the normal controls where there was a very significant correlation between the plasma concentrations of LH and those of the free α subunit. Such quantitative correlations between HCG-β and the free α subunit in our patients, between LH and the free α subunit in our normal controls, are compatible with the concept of a common genetic regulation linking the α and the β subunits in the synthesis of the glycoproteic hormones, the production of the β subunit being the rate-limiting step.

Our observations deserve a last word of

comment: HCG-β and the excessive H-α concentrations presumably reflect an ectopic secretion; according to the "endocrine cell" hypothesis the ectopic hormones are secreted by cells which have some endocrine properties under normal conditions (cells of the APUD series) [11, 12]; the absence of predisposition according to the pathology in our patients does not support this hypothesis as far as HCG and its subunits are concerned.

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Therapeutic Effects of Cysteine Adducts of α,β-Unsaturated Aldehydes on Ehrlich Ascites Tumor of Mice. II*

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Abstract—*The antitumor activity of a cysteine-crotonal and a cysteine-hydroxypentenal adduct was studied against Ehrlich ascites tumor of mice. When female animals were treated with daily doses* (3rd–7th day *following tumor transplantation*) *of* 2.5, 5.0, 10.0 *and* 20.0 mg/10 g *body weight of the crotonal adduct* 30, 30, 36 *and* 33% *survived the* 90th *test day completely free of ascites and solid tumor. The remaining animals responded with an increase of survival time of* 18, 107, 98 *and* 123%. *Male animals receiving daily doses of* 2.5, 5.0 *and* 10 mg/10 g *body weight of the crotonal adduct showed increase of survival time of* 78, 171 *and* 108% *but in none of the animals tumor growth was completely prevented.*

A similar dependence of the therapeutic action on the sex of the treated mice was observed with the hydroxypentenal adduct. In 40% *of female mice injected with daily doses of* 3.5 mg/10 g *body weight of this adduct tumor growth was completely inhibited and the remaining animals showed a significantly prolonged survival time of* 137%. *Male mice treated under equal conditions with the hydroxypentenal adduct responded with an increase of survival time of only* 20%. *The lowest therapeutic active dose of the hydroxypentenal adduct is between* 0.9 *and* 1.75 mg/10 g *body weight.*

Compared on a molar basis crotonal bound to cysteine is 6–11 *and hydropentenal at least* 22 *times less toxic than in the free form. The results indicate that adducts prepared by condensing of* L-*cysteine with α,β-unsaturated aldehydes inhibit growth of Ehrlich ascites cells in mice. Tumor cells growing in female animals, however, are much more susceptible to the compounds than cells growing in male animals.*

INTRODUCTION

IT HAS been reported that the reversible binding of α,β-unsaturated aldehydes with antitumor activity to cysteine offers a promising means of a prolongated action of these aldehydes *in vivo*. Thus it was shown that an adduct prepared by binding of hydroxypentenal (HPE) to cysteine possesses a pronounced therapeutic effect on the growth of EATC in mice [1, 2]. In preliminary experiments it was also found that the adduct prepared by binding of one mole crotonal to two moles cysteine has anticancer activity [1]. In the present paper we report on a more extended study of the latter compound and on some additional experiments conducted with the HPE-cysteine adduct.

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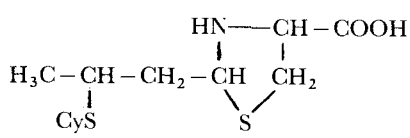
MATERIAL AND METHODS

Experimental animal. NMRI mice of own stock; both sexes; body weight at the beginning of experiment: 20–25 g.

Tumor. Ehrlich ascites tumor, strain Lettré-Heidelberg; harvested 8 days after implantation.

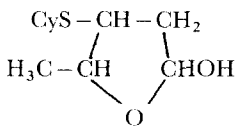
Reagents. The reaction of cysteine with α,β-unsaturated aldehydes and the properties of the so-formed adducts were described in an earlier paper [2]. For the experiments described in this paper, the following adducts were used:

1. Cysteine–crotonal-2:1 adduct (2, 3-trans-butenal) mol. wt: 294; white, hygroscopic powder; m.p.: 168–171° (dec.).



Cysteine–hydroxy–pentenal-1:1-adduct

mol. wt: 221; white, very hygroscopic powder; m.p.: 145° (dec.).



Both the compounds were prepared as zwitter ions. For injection the desired amount was dissolved in 0.9% saline.

Method

For standard implantation approximately 2×10^7 cells (diluted 1:1 in 0.2 ml isotonic NaCl solution) per animal were administered intraperitoneally. Experimental and control animals received $4–5 \times 10^5$ EATC per 10 g of body weight delivered intraperitoneally in 0.25 ml isotonic NaCl. The number of implanted cells was determined before each experiment by means of hematocrit and it was made sure that each animal received the same quantity of cells in relation to its body weight.

For statistical evaluations the bilateral Student's *t*-test was used.

RESULTS

Toxicity

The high toxicity of α,β-unsaturated aldehydes is diminished to a great extent by binding of the aldehydes to L-cysteine [1]. In mice the LD_{50} of a single intraperitoneally dose of hydroxypentenal or crotonal is 0.8 and 1.6 mg respectively per 10 g of body weight [3], cysteine adducts exhibit a LD_{50} which is higher than 45 mg per 10 g body weight (Table 1) [4]. Comparing the toxicity on a molar basis it results that in the cysteine bound form crotonal is 6–11 and hydroxypentenal at least 22 times less toxic than in the free form.

Therapy with the cysteine–hydroxypentenal 1:1 *adduct*

Separate groups of female and male mice bearing the Ehrlich ascites tumor were treated on days 3–7 following transplantation with various doses of the adduct and observed for 90 days (Table 2). Growth of ascites and solid tumor was completely prevented in 40% of the female mice by a dose of 3.5 mg per 10 g of body weight. The remainder of the animals showed a highly significant increase of the survival time of 137% [1]. The same dose administered to male mice could not prevent tumor growth and produced only a 20% extension of the survival time. Doses of 0.9 mg per 10 g of body weight were ineffective in both male and female animals. According to previous results [1] female animals treated with 1.75 mg/10 g of body weight responded with highly significant inhibition of tumor growth. This indicates that for the female animals the lowest therapeutic active dose is in the range of 0.9–1.75 mg per 10 g of body weight.

Therapy with the cysteine–crotonal 2:1 *adduct*

Table 2 compares the therapeutic effectiveness of increasing doses of the adduct against Ehrlich ascites tumor cells growing in female and male mice. In the female group 30, 30, 36 and 33% respectively of the animals were tumor-free on the 90th day when treated with doses of 2.5, 5.0, 10.0 and 20.0 mg/10g of body weight on days 3–7 after transplantation. In the remainder of the animals tumor growth was clearly delayed and the survival time was increased by 18, 107, 98 and 123% respectively. In male mice the adduct proved to be considerably less effective in respect to complete prevention of tumor growth. Thus daily doses of 2.5 and 5.0 mg per 10g of body weight indeed extended the survival

Table 1. Toxic effects of cysteine-HPE 1:1 *adduct and cysteine–crotonal* 2:1 *adduct on NMRI mice (male, 20–25 g of body weight*

Substance	Single dose	Aldehyde bound	Animals Treated	Animals Survivors
HPE-	45	18	5	4
adduct	22	9	5	5
Crotonal-	90	18	10	0
adduct	45	9	10	10
	20	4	10	10

Single dose: mg of adduct per 10 g of body weight, administered intraperitoneally;
Aldehyde bound: mg of HPE or crotonal, present in the adduct;
Survivors: 24 hr after administration.

Table 2. Therapeutic effect of five doses of cysteine adducts, every 72, 96, 120, 144 *and* 168 hr *after implantation with* 4–5 × 10^5 *EATC*/10 g *of body weight*

Controls		Therapeutic animals							
No. of animals	Average survival period ($\pm s_x$)	No. of animals	Died before 90th day (1)	Average survival period ($\pm s_x$)	Extension (%)	Survived 90th day (2)	Survived 90th day (3)	Substance (cysteine-adduct)	Dose mg/10 g body weight
10 f	17.3 (3.8)	9 f	9	17.9 (3.9)	3	0	0	HPE 1:1	0.9
10 f	22.9 (4.6)	10 f	5	54.3 (21.5)	137	4	1	HPE 1:1	3.5
10 m	17.7 (4.3)	10 m	10	21.3 (5.7)	20	0	0	HPE 1:1	3.5 [1]
20 f	23.4 (5.8)	20 f	12	27.7 (19.7)	18	6	2	Crotonal 2:1	2.5
20 f	20.6 (6.4)	20 f	12	42.7 (26.8)	107†	6	2	Crotonal 2:1	5.0
20 f		19 f	9	40.7 (21.3)	98†	7	3	Crotonal 2:1	10.0
10 f	17.3 (3.4)	9 f	6	38.7 (27.9)	123*	2	1	Crotonal 2:1	20.0
		10 m	10	31.6 (22.4)	78	0	0	Crotonal 2:1	2.5
10 m	17.7 (4.3)	10 m	9	41.9 (23.1)	171*	(A)	0	Crotonal 2:1	5.0
		9 m	6	36.8 (20.1)	108†	0	(B)	Crotonal 2:1	10.0

(1) Ascites and solid tumor.
(2) Ascites and solid tumor-free.
(3) Ascites tumor-free, however, with solid tumor.
($\pm s_x$) = standard deviation.
m = male; f = female; * = significant; † = highly significant.
(A) = One animal died at 78th day, free from ascites and solid tumors; cause of dead unexplainable.
(B) = Three animals were certainly free from ascites tumors, but died, between the 48th and 63rd day after implantation from exceedingly developed solid tumors.

time by 78 and 171% but none of the animals was completely free of tumor on the 90th day. A daily dose of 10 mg/10 g of body weight did completely prevent growth of the ascites tumor in 33% of the treated animals. These animals, however, died approximately two months after transplantation from fast developing solid tumors growing at the side where the peritoneum was punctured.

DISCUSSION

The results described give further support for the concept that the reversible binding with cysteine is a promising way of increasing the therapeutic effectiveness of tumor inhibitory α,β-unsaturated aldehydes.

The use of cysteine adducts for tumor therapy has the advantage that the adducts are several times less toxic than the free aldehydes. It is assumed that the cysteine adducts are storage forms which slowly and gradually release the bound aldehyde in the tumor cells and thus provide a prolonged action. The liberated aldehyde in turn reacts with SH-groups of functional importance for DNA synthesis and thereby prevents growth of tumor cells. It has been shown by histochemical methods that incubation of EATC with a cysteine adduct leads to a release and concomitant binding of the aldehyde to proteins in nuclear area [5]. The therapeutic effectiveness depends upon the stability of the adducts. Thus the very stable cysteine–acrolein 2:1 adduct and the cysteine–HPE 2:1 adduct are therapeutically ineffective [1] while the less stable cysteine–HPE 1:1 adduct and the cysteine–crotonal 2:1 adduct are therapeutically effective. Both adducts completely prevent or significantly inhibit growth of EATC in female mice. Treated with the HPE adduct (1.75–14.0 mg/10 g of body weight) 22–40% of the animals remained macroscopically free from ascites and solid tumor while in the remainder of the animals the survival time was extended up to 289%. Similar results were found for the crotonal adduct (2.5–20.0 mg/10 g of body weight) which completely prevented tumor growth in 22–35% of the animals and delayed it in the remainder of the animals up to 123%. In male animals the adducts proved to be much less therapeutically effective.

Depending on the dose male mice responded with an increase of survival time between 78 and 171% when injected with the crotonal adduct. With the HPE adduct the extension of survival time was 20%. The considerable lower therapeutic effectiveness of the adducts in male animals may be due to an increased rate of detoxification and excretion, to a lower activity of an enzyme catalyzing the liberation of the aldehyde in the tumor cells or to a hormonally controlled interaction between the host and the tumor cells. Up to now, however, no certain decision can be made which factors actually are responsible for the sex difference.

The results confirm the previous findings [1] that cysteine adducts of α,β-unsaturated aldehydes possess antitumor activity. The therapeutic effectiveness against Ehrlich ascites tumor of mice strongly depends on the sex of the animals.

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Synergism and Schedule Dependent Cytotoxicity of Cyclophosphamide and CCNU in Experimental Cancer Chemotherapy*

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Abstract—*A synergistic degree of cell killing was obtained with cyclophosphamide and CCNU in the L1210 leukaemia, the Lewis lung carcinoma and the mouse C22LR osteosarcoma. Drug synergism was also demonstrated in both resting and rapidly proliferating haemopoietic stem cells. By scheduling the 2 drugs we attempted to obtain selective cell kill of tumour cells over vital normal cells. The optimum antitumour schedule was found to be that when the drugs were given simultaneously. This tumour based schedule however, proved to be the most toxic for bone marrow stem cells as well. Therefore, the clinical relevance of this synergistic drug combination is expected to be limited.*

INTRODUCTION

THERE is ample evidence at both the preclinical and clinical level that combination chemotherapy is more effective than single drug treatment. The experimentalist should try not only to discover new therapies but to investigate also how to use existing drugs more effectively: what are the optimal conditions for their usage and what are the underlying mechanisms leading to improved therapy. While there are many perspectives on chosing combinations of agents, there is but one goal: that being to maximize the killing of malignant cells while minimizing the killing of normal cells.

Drug schedule design can be devised on the basis of toxicological, cell kinetic, biochemical and pharmacological considerations. Our selection of agents, however, has been empirical. The two-drug combination of cyclophosphamide (Cyclo) plus a nitrosourea was found to be therapeutically potentiating against various spontaneous or transplanted murine tumour lines [1]. However, if a combination of two chemotherapeutic agents produces an increased cytotoxic effect on tumour tissue with a concomitant intensified toxicity to host tissues, then the therapeutic gain of a so-called synergistic drug combination will be negligible.

Therefore, we have investigated whether Cyclo in combination with CCNU was synergistically active in a range of animal tumours as well as in the most critical normal tissue, bone marrow stem cells.

Stimulated by our 5-fluorouracil–CCNU findings [2], our approach was to investigate whether we could improve the therapeutic ratio by drug scheduling: CCNU was given simultaneously with or at a time interval of 24 hr before or after Cyclo.

If several experimental tumour lines show a similar response to a particular drug schedule, it is warranted to extrapolate the results to clinical treatment. Moreover, the reactions of the bone marrow cells to cytostatic drug treatment in mice and in man are not essentially different. Therefore, the conclusions of our preclinical animal work will allow us to extrapolate them to cancer patients with some degree of confidence.

The purpose of this study was to investigate the effect of Cyclo–CCNU combination chemotherapy in widely different tumour cell lines as well as in bone marrow and secondly to determine whether, by suitable drug scheduling,

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a difference between malignant and normal tissues could be therapeutically exploited.

MATERIAL AND METHODS

Material

The Lewis lung carcinoma was maintained by s.c. serial transplantation in the C57Bl/Ka mouse and transplanted into (C57Bl/Rij × CBA/Rij)F1 mice (hereafter called BCBA F1) for chemotherapy experiments. The C22LR osteosarcoma studies were performed in BCBA F1 mice with tumour of passages 83 and 84 from frozen samples of passage 77. The volume doubling time of both solid tumours when measured at between 200–800 mm^3 is 2.5 days [2]. The L1210 leukaemia was obtained from Dr. C. von Essen, Yale University, New Haven, CT, in 1969. The leukaemia was initially maintained in our laboratory by weekly transfer in (C57Bl/Rij × DBA/2)F1 hybrid mice. Since 1975 (BALB/c × DBA2)F1 (hereafter called CD2 F1) hybrids have been used. In 1973 the leukaemic cells were adapted to growth *in vitro* and maintained by alternating *in vivo* and *in vitro* transfer. The doubling time of our exponentially growing L1210 *in vitro* is 10 hr. CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea NSC-79037, was kindly supplied by Laboratoire Roger Bellon S.A., Neuilly sur Seine, France. The drug was suspended in 2% carboxymethyl-cellulose. Cyclophosphamide, NSC-26271, was a gift from Asta-Werke, Brackwede, Germany. Both agents were injected i.p. and the maximum dosages used were less than 0.5 of the LD_{10} dosages, except for the experiments shown in Tables 1 and 2.

Table 1. The effect of treatment of Lewis lung carcinoma with different drug doses expressed as growth delay

	CCNU mg/kg		
		10	20
Cyclo mg/kg	0 ± 0.5	2.7 ± 0.8	4.1 ± 0.8
100	6.8 ± 1.7	8.1 ± 1.8	14.8 ± 2.0
200	16.4 ± 1.6	2/5 NED	4/5 NED

BCBA F1 mice were inoculated s.c. with 10^6 Lewis lung tumour cells on day 0. Treatment was given on day 10. NED indicates no evidence of disease on day 62.

Methods

Cells from a tumour suspension were inoculated s.c. or i.m. in mice [2]. Treatment with Cyclo and CCNU was either simultaneously or sequentially: CCNU 24 hr before (CCNU → Cyclo) or 24 hr after Cyclo injection (Cyclo → CCNU). Tumours were measured twice weekly with calipers. The endpoint of treatment of both solid tumour lines was growth delay in days: the displacement in time between the growth curve of the control group and the growth curve of the tumours recurring after treatment. The Student's *t*-test applied to individual tumour growth delay data was used for the evaluation of the results of treatment with the different combinations.

From an exponentially growing L1210 in *in vitro* culture, 10^5 cells were injected i.p. in CD2 F1 mice. The percentage increase in lifespan (% ILS) was used as an index of antitumour activity and was based on the median day of death of the group. The effect of treatment on L1210 leukaemia was also investigated by a combined *in vivo* and *in vitro* technique [3]. In treated leukaemic mice, the number of surviving L1210 cells was determined by cloning *in vitro*. The surviving fraction was calculated as the ratio of the number of donor leukaemic colony forming units (CFU) per femur of a treated group to that of a control group.

To assess the activity of drugs against normal cells, we determined the survival of resting haemopoietic stem cells by the spleen colony technique of Till and McCulloch [4]. After treatment with a cytostatic drug, the normally resting stem cells will be recruited into cycle and this may result in increased drug sensitivity. Therefore, we also investigated the effect of treatment on recruited, rapidly proliferating normal bone marrow stem cells [5]. The survival of resting and proliferating CFU was calculated as the ratio of the mean number of CFU per donor femur and per spleen, respectively, of treated to that of simultaneously assayed control mice.

Table 2. The effect of high dose treatment with cyclophosphamide and CCNU in osteosarcoma: the change in tumour volume doubling time (TVD) of the recurrent tumours

Treatment	Growth delay (days)	T.V.D. (days)
Control	Mean ± S.E.: 0 ± 0.4	2.5
Cyclo and CCNU	Individual: 17, 46, 55, NED, NED	2.5, 8, 12

One million osteosarcoma cells were injected s.c. into BCBA F1 male mice on day 0. Treatment with Cyclo 100 mg/kg i.p. and CCNU 20 mg/kg i.p. was given on day 7. NED indicates no evidence of disease on day 121.

RESULTS

Three different endpoints were used in our experiments: growth delay, lifespan and cell survival. Tumour growth delay is, under specific conditions to be discussed, correlated with tumour cell kill; therefore, if the joint action of agents is greater than the algebraic sum of their individual effects, we interpret this as drug synergism. The same definition is used for the results of treatment of the leukaemic mice. If the percentage increase in lifespan (%ILS) is greater than the algebraic addition of the response achieved with either drug alone, the drug combination acts synergistically. For the cell survival experiments, synergism is found when the observed surviving fraction is smaller than the product of the individual survival fractions.

The effect of single drug treatment and the results of combination chemotherapy on mice with s.c. inoculated Lewis lung tumour cells are shown in Table 1. The expected growth delay of the drug combination CCNU 20 mg/kg and Cyclo 100 mg/kg is 4.1 plus 6.8 or approximately 11 days. However, the observed growth delay is 14.8 days. Therefore the therapeutic synergistic effect with these drug dosages is about 4 days. For the highest dose combination used, the difference between expected (20.5 days) and observed growth delay is more than 40 days, since 4 out of 5 mice showed no evidence of disease at day 62.

Data on the Cyclo + CCNU combination for the C22LR osteosarcoma are presented in Table 3 and for the L1210 leukaemia in Tables 4 and 5. Note that when more effective drug dosages are used the standard error of the mean tumour growth delay will increase (Table 3). Table 2 shows an extreme example: the growth delay varies between 17 and 55 days. This variation in growth delay is caused by a dramatic change in volume doubling time of the tumours in the treated animals; only one out of 3 recurrent tumours has the original doubling time of 2.5 days.

The influence of drug scheduling (Cyclo → CCNU and CCNU → Cyclo) on the L1210 leukaemia, the Lewis lung carcinoma and the C22LR osteosarcoma is shown in Tables 4, 5, 6 and 7. All experiments (except experiment No. C 128 in Table 4) show the same pattern: the optimal drug sequence for tumour treatment is when both drugs are given simultaneously (Cyclo + CCNU).

In order to evaluate therapeutic gain, we also investigated the effects of both drugs on resting and proliferating bone marrow stem cells (Table 8). The agents were given either simultaneously or in different sequences. The simultaneous, Cyclo + CCNU, treatments are synergistically active and, in comparison with the sequential treatments the most effective ones. Therefore the most toxic drug schedule is when both drugs are given at the same time (Cyclo + CCNU) in both states of bone marrow proliferation.

DISCUSSION

The observed effect of treatment with Cyclo + CCNU on the Lewis lung carcinoma, the C22LR osteosarcoma and the L1210 leukaemia is more than expected from the effects of treatment with single drugs. This synergism of Cyclo with one of the nitrosoureas has been demonstrated in the AKR lymphoma [6], the KHT fibrosarcoma [7] and the Lewis lung carcinoma [8], as well as three different experimental colon tumour lines [9]. Therefore, it is frustrating to discover that the integration of these preclinical findings into clinical cancer treatment have not resulted in therapeutic improvement. Two negative treatment reports [10, 11], together with our own experience with 12 patients with advanced renal cell carcinoma (unpublished observations) do not support the clinical application of the preclinical findings of Cyclo-nitrosourea synergism.

The disappointing therapeutic results may be the result of the concomitant increased toxicity of the drug combination. Although a group of investigators in St. Louis showed in preliminary data an enhanced cell kill of L1210 cells over normal haemopoietic stem cells with 1,3-bis(2-chloroethyl)-1-nitrosourea and Cyclo [12], our data seem less promising. The synergistic effect on our two solid tumour lines (Tables 1, 3, 6 and 7) and on the L1210 leukaemia (Tables 4 and 5) was accompanied by an increased cell killing effect on bone marrow stem cells (Table 8). Therefore it will be difficult to increase the therapeutic ratio: the enhanced effect of treat-

Table 3. The synergistic effect of treatment with cyclophosphamide and CCNU in osteosarcoma

Treatment	Mean growth delay and standard errors (days)
Control	0 ±0.4
Cyclo 100 mg/kg i.p.	8.6 ±0.9
CCNU 10 mg/kg i.p.	4.1 ±0.8
Cyclo + CCNU	27.1 ±4.0

One million osteosarcoma cells were injected s.c. into groups of 5 BCBA F1 male mice on day 0. Treatment was given on day 7.

Table 4. The influence of drug scheduling with cyclophosphamide and CCNU on the lifespan of leukaemic mice

	% ILS				
Exp. No.	C127	C128	C129	C131a	C131b
Cyclo	12.5	—	—	0	10
CCNU	37.5	—	—	20	20
Cyclo→CCNU	37.5	55	55	20	40
Cyclo+CCNU	87.5	55	111	60	110
CCNU→Cyclo	50	44	44	20	50

L1210 cells were injected i.p. into groups of at least 5 CD2 F1 mice. Treatment was given within 6 days after cell inoculation. A dose of 10 mg/kg CCNU was given i.p. in combination with a dose of Cyclo which varied in the different experiments between 50 and 100 mg/kg i.p. Drug induced deaths in nontumour bearing animals were not observed. Data are expressed in terms of the percentage increase in survival as determined by the equation:

$$\frac{\text{median survival of treated animals (days)}}{\text{median survival of control animals (days)}} \times 100 = \%\,\text{ILS}.$$

Synergism is evident when the observed % ILS is greater than the algebraic sum of the individual % ILS. The effect of single drug treatment was not investigated in experiments C128 and C129.

Table 5. Surviving fraction of L1210 cells treated with cyclophosphamide and CCNU

	Surviving fraction	
Treatment	Experiment I	Experiment II
Cyclo 30 mg/kg i.p.	0.23	0.16
CCNU 10 mg/kg i.p.	0.11	0.18
Cyclo→CCNU	0.03	0.05
Cyclo+CCNU	0.002	0.01
CCNU→Cyclo	0.02	0.02
Calculated effect of combination	0.02	0.03

Synergism is evident when the observed surviving fraction is smaller than the expected surviving fraction calculated by multiplying the individual surviving fractions.

Table 6. The effect of sequential treatment with cyclophosphamide and CCNU in Lewis lung carcinoma

Treatment schedule	Mean growth delay and standard errors (days)	
Control	0 ±0.3	
Cyclo 100 mg/kg i.p.	3.5±0.5	
CCNU 10 mg/kg i.p.	0.5±0.5	
Cyclo→CCNU	12.0±0.6	$P<0.001$
CCNU+Cyclo	19.0±1.6	
CCNU→Cyclo	9.0±0.7	$P<0.001$

Groups of 10 BCBA F1 male mice were inoculated i.m. with 10^6 Lewis lung tumour cells on day 0. Cyclo was given either simultaneously, 24 hr before or 24 hr after CCNU treatment on day 5.

Table 7. The effect of sequential treatment with cyclophosphamide and CCNU in osteosarcoma

Treatment schedule	Mean growth delay and standard errors (days)	
Control	0 ±0.3	
Cyclo 50 mg/kg i.p.	5.0±0.4	
CCNU 10 mg/kg i.p.	4.2±0.7	
Cyclo→CCNU	14.3±0.9	$P<0.005$
CCNU+Cyclo	18.0±0.5	
CCNU→Cyclo	13.6±0.8	$P<0.001$

One million osteosarcoma cells were injected s.c. into groups of 10 BCBA F1 male mice on day 0. Cyclo was given either simultaneously, 24 hr before or 24 hr after CCNU treatment on day 5.

Table 8. Percentage of surviving normal haemopoietic bone marrow stem cells per femur and repopulating spleen in comparison with untreated control mice

	Resting stem cells		Proliferating stem cells		
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. III
Cyclo 50 mg/kg i.p.	47.72	15.70	7.73	37.70	2.22
CCNU 20 mg/kg i.p.	13.02	3.49	0.13	2.83	7.11
Cyclo→CCNU	8.30	1.57	1.87	3.35	0.44
CCNU + Cyclo	2.25	0.1	0.08	0.08	0.15
CCNU→Cyclo	3.82	2.86	0.39	0.82	—
Calculated percentage	6.21	0.55	0.01	1.06	0.16

ment on tumours will always be accompanied by more host toxicity.

In all experimental systems in which less than approximately half the LD_{10} dosages were used, drug synergism was evident. When higher dosages were applied in the solid tumour experiments, we observed an increase in tumour volume doubling time (Table 2). After treatment with nitrosourea, the regrowing tumours may consist of a selection of cells which are more slowly dividing than were the cells of the original tumour [13]. In this situation, it is inappropriate to use the growth delay as an indirect parameter of tumour cell kill. Conclusions on synergism based on tumour regrowth times should only be made when the growth curves of the recurrent tumours are parallel to the control curve and as long as there is a linear dose–response relationship. To avoid deviation from these conditions, we have used low dosages of Cyclo and CCNU. A second artificially induced drug synergism may be produced by an enhanced tumour cell kill through an immunological phenomenon. After intensive treatment a small tumour mass may be further reduced by an immunological attack. If this occurs, the observed synergistic effect of drug treatment should not be interpreted as a result of drug interaction only. In our mouse tumour systems, the Lewis lung carcinoma is immunogenic; the C22LR osteosarcoma, however, is not. Moreover, the Cyclo–CCNU combination shows the more than additive effect on such a variety of cell lines that the explanation for synergism must probably be sought in a biochemical interaction of both drugs. This finding is of theoretical importance, since, in clinical management, the cancer chemotherapist tries to avoid drugs with the same mechanism of action in a drug combination. This policy should not be confused with the principle of alternating drugs or drug combinations in order to circumvent cross resistance. Nevertheless, we have here a very effective drug combination of two agents both of which have alkylating moieties [14].

The combination Cyclo–CCNU acts synergistically on neoplastic as well as normal tissues and there is no simple way to design a treatment schedule in which drugs are given sequentially which may lead to better therapeutic results. Therefore, the clinical application of our findings with this drug combination in whatever schedule given are expected to be disappointing, particularly if a large fraction of sensitive normal cells are in cell cycle. From a theoretical point of view, the combination is extremely interesting, although, for the above reasons, its relevance to clinical practice is limited.

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Tumour-Associated Antigens in Cystic Melanomas*

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Abstract—*Fluid from five cystic secondary melanomas was examined for its effect on the migration of peripheral blood leukocytes from melanoma patients and control donors. Two samples selectively inhibited the migration of melanoma leukocytes. Fractionation showed the mol. wt of the active component(s) to be between* 41,000 *and* 95,000. *Fluid of this type, though not ideal, may be used as a source of antigen in the leukocyte migration technique and presumably in other techniques.*

INTRODUCTION

METHODS for extracting antigens from tumour cells remain imperfect and may distort membrane characteristics peculiar to the tumour, yielding products unrepresentative of the relative proportions or configurations of antigens *in vivo*. Ideally, antigens should be clinically accessible, and not require complex or damaging preparation prior to use.

Partial necrosis of tumours is common, and such areas may undergo cystic degeneration. Fluid from such cystic tumours might be a convenient source of tumour antigens. Previous studies of fluid from a cystic melanoma showed that it reacted in an apparently specific manner with melanoma patients' leukocytes, causing blast transformation of lymphocytes [1] and inhibiting leukocyte migration [2]. The active fraction had a mol. wt. of less than 100,000. The present study was to ascertain how frequently melanoma cyst fluids showed selective activity, and to characterize the active component by using previously proven methods, involving chromatography and electrophoresis e.g. [3, 4,] and [5]. We have therefore applied these techniques to melanoma cyst fluids and tested the fractions for activity in leukocyte migration inhibition assays.

MATERIAL AND METHODS

Leukocyte donors

Peripheral blood was obtained from 32 patients with histologically proven and clinically staged malignant melanomas. Controls [34] were patients with non-malignant diseases and healthy individuals.

Source of antigen

Fluid (0.3–1.0 ml) was obtained by needle aspiration of the melanomatous lymph nodes of four patients and from a cystic local tumour recurrence of one of these patients. Fluids were stored at −20°C until tested.

Preliminary titrations indicated a suitable concentration for the detection of cell-mediated reactivity to be 500 μg protein per ml of leukocyte suspension, and all tests with unfractionated cyst fluid were performed at this concentration. Details of the preparation of leukocytes and the leukocyte migration inhibition assay have been published [6].

Statistical analysis

Groups of four replicate migration areas with and without antigens for each individual were compared using the Mann–Whitney–Wilcoxon U test of ranking. Positive reactivity was where the difference between quadruplicate observations in the presence and absence of antigen was significant at the 5% level. The frequency of positive reactions in each group of leukocyte donors was compared using the chi-squared test.

Fractionation procedures

A flow chart of the procedures employed is shown in Fig. 1.

Gel filtration

Aliquots (0.5 ml) of melanoma cyst fluids

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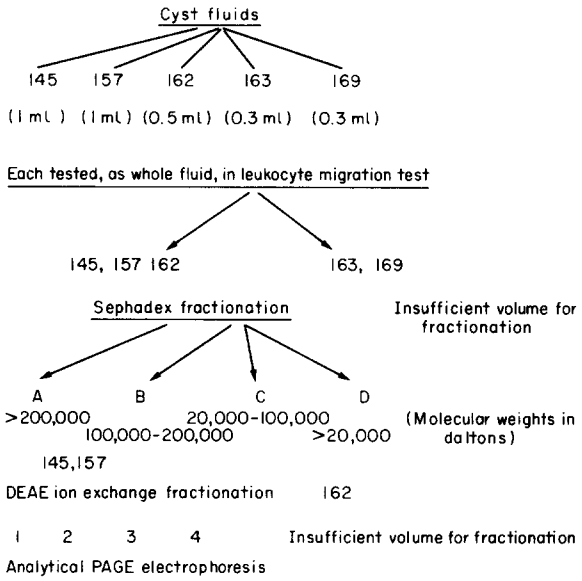

Fig. 1. Separation protocol of melanoma cyst fluids.

(where sufficient was available) were applied to a Sephadex G150 column (2.6 × 40 cm) equilibrated with phosphate buffered saline (PBS). The flow rate was maintained at 30 ml/hr and all operations were performed at 4°C. Fractions (5 ml) were collected into pools A–D, as shown in Fig. 2 and concentrated by dialysis against

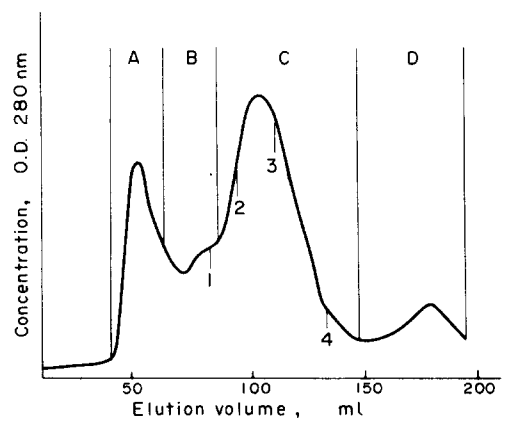

Fig. 2. Elution profile of melanoma cyst fluid No. 162 from Sephadex G150. Molecular weight markers. 1. Human γ-globulin (180,000). 2. Transferrin (80,000). 3. Haemoglobin (65,000). 4. Trypsin (25,000).

Aquacide II (Calbiochem, London) to the original sample volume. They were then extensively re-dialysed against PBS and stored at −20°C. The column was calibrated by elution of proteins of known mol. wt (human γ-globulin, 180,000; transferrin, 80,000; haemoglobin, 65,000; and trypsin, 25,000).

Ion exchange chromatography

Pooled fractions B and C from Sephadex G150 chromatography were dialysed against 0.005 M Tris–HCl (pH 8.0) and 0.3 ml aliquots applied to a DEAE-52 cellulose column (1.5 × 25 cm) equilibrated in the same buffer. Fractions (5 ml) were eluted with a linear gradient of increasing salt concentration from 0.005 M Tris–HCl to 0.75 M NaCL in 0.005 M Tris–HCl (pH 8.0). Flow rate was maintained at 15 ml/hr and fractions pooled (1–4) as shown in Fig. 3, dialysed against PBS and concentrated to the original sample volume by dialysis against Aquacide II. Samples were extensively re-dialysed against PBS. All procedures were performed at 4°C. The amount of fluid in the fractions was too small to allow determination of protein concentration; aliquots of 10 μl were added to each well in the leukocyte migration tests.

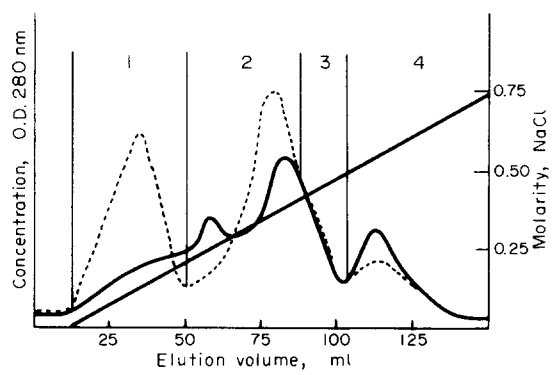

Fig. 3. Elution profile of melanoma cyst fluids 145 (broken line) and 157 (full line) from DEAE 52.

Analytical polyacrylamide gel electrophoresis

This was performed as described previously [7]. Electrophoresis was continued at 3 mA per tube, until bromophenol blue, added as a tracker dye, had passed to the bottom of the gel. Each gel was stained for protein with 0.5% Coomassie Blue. Molecular weight was estimated by comparison of the migration of proteins of known molecular weight relative to bromophenol blue.

Protein concentration

Approximate protein concentrations of the whole cyst fluids and Sephadex fractions were determined by absorption at 280 nm.

RESULTS

Whole cyst fluids

The luekocytes of 16 out of 29 melanoma patients (55%) and 6 out of 26 control individuals (23%) were selectively inhibited by the cyst fluids (melanoma leukocytes, 29/56 tests positive, 52%; control leukocytes, 12/50 tests positive, 24%) ($P = <0.01$), as seen in Table 1.

Analysis of the results on the basis of the reactions induced by indivudual fluids showed

Table 1. *Effects of whole cyst fluids on migration of leukocytes from melanoma patients and control donors*

Cyst fluid	Leukocyte donor	Inhibition alone Individuals positive/ total tested	(%)	Significance
145	Control	3/11	(27)	ns
	Melanoma	4/12	(33)	
157	Control	0/10	(0)	$P<0.05$
	Melanoma	5/11	(45)	
162	Control	4/13	(31)	ns
	Melanoma	6/12	(50)	
163	Control	4/8	(50)	ns
	Melanoma	7/11	(64)	
169	Control	1/8	(12.5)	$P<0.05$
	Melanoma	7/10	(70)	
Total	Control	12/50	(24)	$P<0.01$
	Melanoma	29/56*	(52)	

ns = not significant.
Positive tests in melanomas compared with those in controls by the chi squared test.
*The total number of tests on melanoma patients and controls exceeds the total number of individuals in each category as some were tested on more than one occasion or against more than one fluid.

that all antigens inhibited melanoma leukocytes. While two, 157 and 169, showed significant selective inhibition, the other three showed non-selective reactivity, inhibiting control leukocytes, such that differences between tests and controls were not significant. Enhancement was encountered only rarely in these studies and did not alter the patterns of reactivity observed.

Leukocytes from Stage I melanoma patients were more often inhibited than those from Stage II patients (Table 2).

Table 2. *Effects of cyst fluids on migration of leukocytes from melanoma patients and control donors; comparison by stage for selectively active fluids* 157 *and* 169 *and non-selective fluids* 145, 162 *and* 163

	Whole cyst fluid Individuals positive/total tested (%)	
	157 and 169	145, 162, and 163
Stage I	5/6 (83)*	8/16 (50) ns
Stage II	7/15 (46)*	6/15 (40) ns
Stage III	none tested	3/4 (75) ns
Total melanomas	12/21 (57)*	17/35 (48) ns
Controls	1/18 (5)	11/32 (34)

Positive tests in melanomas compared with those in controls by the chi squared test.
ns = not significant.
*Significant ($P<0.01$).
Note that the total number of tests on melanoma patients and controls exceeds the total number of individuals in each category as some were tested on more than one occasion or against more than one fluid.

Fractionated cyst fluid

Sufficient cyst fluid was available from two "non-selective" samples (145 and 162) and one selectively active sample (157) to allow fractionation on Sephadex G150. All gave similar elution profiles (O.D. 280 nm. trace), a representative sample of which is presented in Fig. 2. In migration studies, fractions from the three samples showed similar patterns of activity, and are therefore presented together in Table 3. Pigmented material was observed in two regions, corresponding to mol. wt of approximately 200,000 and 20,000.

A total of 24 melanoma patients' and 18 control leukocytes were studied. Material from region A (mol. wt >200,000) of fluids 145 and 157 was available for assessment. Both induced a similarly low frequency of migration inhibition in control and melanoma patients' leukocytes was noted in fraction C (mol. wt 20,000–100,000) (Table 3). Fraction B also inhibited more melanoma patients' leukocytes than con-

Table 3. Effects of Sephadex fractions on migration of leukocytes from melanoma patients and control donors

Fraction	Leukocyte donor	No. Total	Inhibited/ tested (%)	Significance
A				
(Mol. wt >200,000)	Control donors	2/12	(17)	ns
	Melanoma patients	3/17	(18)	
B				
(Mol. wt 100,000–200,000)	Control donors	1/23	(4)	ns
	Melanoma patients	7/31	(23)	
C				
(Mol. wt 20,000–100,000)	Control donors	1/23	(4)	$P<0.01$
	Melanoma patients	11/30	(37)	
D				
(Mol. wt <20,000)	Control donors	5/22	(23)	ns
	Melanoma patients	3/28	(11)	

Positive tests in melanomas compared with those in controls by chi-squared test.
ns = not significant.
Note that the total number of tests on melanoma patients and controls exceeds the total number of individuals in each category, as some were tested on more than one occasion or against more than one fraction.

trol leukocytes, but the difference in reaction frequency was not significant ($0.10>P>0.05$). Preferential activity was thus induced in the previously non-selective samples by Sephadex G150 chromatography. Fraction D (mol. wt <20,000) caused a low frequency of migration inhibition in both leukocyte donor groups. Activity was not associated with the fractions containing pigmented material. At the concentration of antigen used in the tests no fraction showed gross toxic effects, i.e. frequently inhibiting leukocytes of both melanoma patients and controls.

Because of the apparent preferential activity of fraction pools B and C the residue of that material from fluid 157 was pooled and further separated on DEAE 52 ion exchange columns. As a control, the comparable regions from the non-selective fluid 145 were similarly separated. Elution profiles of the samples were similar, though not identical (Fig. 3). Analysis of the four DEAE 52 fractions from each sample by polyacrylamide gel electrophoresis revealed a heterogeneous mixture of between two and six bands in each fraction, with mobilities between 0.43 and 0.81 relative to bromophenol blue. These correspond to mol. wt of 26,000–110,000 although individual fractions generally contained proteins of more restricted molecular weight range. Migration studies were performed using these fractions as antigens. None of the fractions inhibited the migration of control leukocytes (8 individuals, 36 tests), but inhibitions were detected in 10/41 tests with melanoma patients' leukocytes (4 out of 9 patients). Inhibitory activity was not confined to any one fraction and was observed in both samples.

DISCUSSION

The studies of these cyst fluids indicated preferential inhibition of the migration of melanoma patients' leukocytes relative to control donors' leukocytes. Analysis of the individual preparations, however, showed only two samples to have significant selective activity. These active samples were from tumorous lymph nodes (157 and 169), as were two of the three non-selective samples (145 and 163); the third being from a locally recurrent nodule from one of these (145). The relatively high number of control donors having leukocytes which were inhibited by contact with these three non-selective fluids present problems of interpretation. The unseparated fluids were characterized by their capacity frequently to inhibit leukocytes from both melanoma patients and control donors, perhaps indicating the presence of toxic materials which were not, however, demonstrated in any of the fractions.

The observation that cyst fluid contains materials with some degree of tumour specificity is in accordance with previous reports [1, 2], although care must be taken in the selection and assessment of each sample prior to extensive

investigation. Attempts to purify the active materials in these fluids were hindered by the small amounts available. Active fractions could be separated by gel filtration on Sephadex G150. Maximum activity was present in a fraction containing materials of mol. wt 20,000–100,000, and further separation suggested a mol. wt between 41,000 and 98,000. Selective activity persisted after DEAE 52 fractionation, but reactions were less frequent, perhaps reflecting the importance of using an optimum dose of this material. It is, however, likely that the active materials within these cyst fluids are a heterogeneous group of molecules of variable characteristics, since cyst formation results from a degenerative process which may lead to the breakdown of antigenic entities *in vivo*. Such autodigestive phenomena in association with necrosis are well recognized.

The range of molecular weights determined for the active fractions in this study is in agreement with those isolated from melanomas by various procedures. Thus, a fraction active in inducing delayed hypersensitivity from sonicated melanoma membrane preparations, isolated by gel filtration and polyacrylamide gel electrophoresis, had a mol. wt of 38,000 [8]. Similarly, 3M KCL extracts of melanoma, following gel filtration [5], resulted in similar molecular weight fractions to those described here.

When fractions were separated by chromatography, selective activity was revealed in previously non-selective samples. Such induction of reactivity by purification has been described previously [5, 8] and suggests the presence of blocking factors or inhibitors in the cyst fluids. Unfortunately, insufficient material was available to permit recombination of fractions in order to investigate this phenomenon further.

Cyst fluid is thus a practical though not ideal material for the study of sensitisation to tumour antigens. It has the advantage of clinical accessibility and requires no complex or damaging preparative manoevres prior to use. A direct comparison of active materials derived from such fluids with materials released from tumour membranes by KCL, sonication, detergents, or enzymic digestion, would be of particular interest.

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Dehydroepiandrosterone and Androstenediol in Human Primary Breast Tumours*

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Abstract—*Dehydroepiandrosterone (DHA) and 5-androstene-3β,-17β-diol have been measured in a series of primary human breast tumours. All the tissues contained significant quantities of DHA and there was a negative correlation of these values with the age of the patient. Only just over one half of the tumours contained detectable levels of androstenediol but there was an over-all correlation of this steroid with the DHA, although concentrations of DHA were generally much greater. No relationship between the steroid content of the tumour and the oestrogen receptor levels was established although receptor was not measurable in the relatively small number of tumours which contained high levels of either steroid. Quantities of endogenous C_{19}-steroids would seem to be high enough to exert some influence on the activity of the oestrogen receptor* in vivo.

INTRODUCTION

THE RELATIONSHIP of C_{19}-steroids to breast cancer is not clearly understood although the evidence from studies of urinary steroid excretion [1–4], plasma steroid assays [5–6] and data on the paraendocrine behaviour of some tumours [7–9] would indicate that involvement of dehydroepiandrosterone (DHA) or its sulphate is of major importance.

More recently [10–11] it has been shown that androgens can directly displace oestradiol from the oestrogen receptor protein present in a number of breast tumours and may exert an influence on the activity of the tumour cells. In order to determine whether C_{19}-steroids are present in sufficient concentration within the tissue itself to affect tumour growth or activity DHA and 5-androstene-3β, 17β-diol (androstenediol) levels in a series of human primary breast tumours have been determined using a gas chromatographic–mass spectrometric technique previously described [12] and are compared to the level of cytoplasmic oestrogen receptor in the tissues.

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MATERIAL AND METHODS

Tissues

Primary breast tumours were collected by the Department of Surgery, University of Liverpool, stored dry at −20°C and transported to the Tenovus Institute, Cardiff in dry ice. A 15% (w/v) homogenate of the tumours was made in 10 mM Tris–HCl buffer for assay of oestrogen receptor and portions of the total homogenate were stored at −20°C until assayed for steroid content.

Assay of DHA and androstenediol

The details of the method of analysis have been published in full [12]. Briefly the procedure consisted of equilibration of homogenate with internal standards to monitor recovery of steroid followed by extraction of lipid materials with acetone/ethanol. Much of the non-steroidal lipid was removed by solution of steroid in hot aqueous $CaCl_2$ [13], and the partially purified, dried residue derivatised by the addition of bis-trimethylsilyl acetamide (BSA). After standing at room temperature overnight, aliquots (1.5 μl) of the BSA were injected into a g.l.c. column containing 3% OV-17 and the eluate passed into a Varian MAT 731 mass spectrometer set up for high resolution single ion monitoring. Standards were run with every set of extractions and results

expressed as ng of steroid per g wet weight of tissue.

Oestrogen receptor assay

The oestrogen receptor content of the tissues was measured by methods routinely used in the Institute. The procedure involved the incubation of portions of a high speed supernatant with varying amounts of [^{3}H]oestradiol with separation of free and bound steroid by dextran coated charcoal. Binding site concentration was determined using equations derived from the Law of Mass Action and processed by computer using a Newton–Raphson iterative curve fitting technique [14]. The results were expressed as fmole of high affinity oestradiol binding per mg cytosol protein.

RESULTS

The assay procedures have been shown to measure specifically non-conjugated DHA and androstenediol [12] and in this series a large number of primary breast tumours have been analysed for both steroids. All the tissues examined were found to contain measurable quantities of DHA in levels ranging from 2.5 to 466.0 ng/gm tissue. There was a highly significant ($P<0.001$) correlation between the DHA content of the tumour and the age of the patient, younger women generally bearing tumours with considerably higher amounts of DHA than older women (Fig. 1). The negative correlation was greater when the values of DHA were expressed as logarithms ($R=-0.44$).

In contrast androstenediol was measurable in just over one half of the tumours, 42% of the tissues contained less than 1 ng steroid/gm. The range of values in those tumours where androstenediol was present was much lower than those of DHA, up to 40 ng/gm (Fig. 2). No significant correlation was found between androstenediol levels in the tumour and the age of the patient although the highest amounts were found in tissues taken from younger patients (Fig. 2). Despite this there was a very significant ($P<0.001$) positive correlation ($R=0.47$) between DHA and androstenediol levels in tumours where both these steroids were measured in one extract (Fig. 3).

Since the correlation of DHA with age of patient could be due to the menopausal status of the women it is of interest that amounts of DHA, but not of androstenediol, were significantly ($P<0.05$, Mann–Whitney U test) higher in tumours from women who were pre-menopausal. The range of values were 5.4–300.0 ng/g in this group compared to 3.2–81.8 ng/g in tissues from women known to be at least two years past the menopause.

No significant correlations were seen between the levels of DHA or of androstenediol in the whole tumour and the amount of available oestradiol receptor sites in the cytoplasm. As can be seen from Figs. 1 and 2, tumours classified as receptor negative or positive (greater than

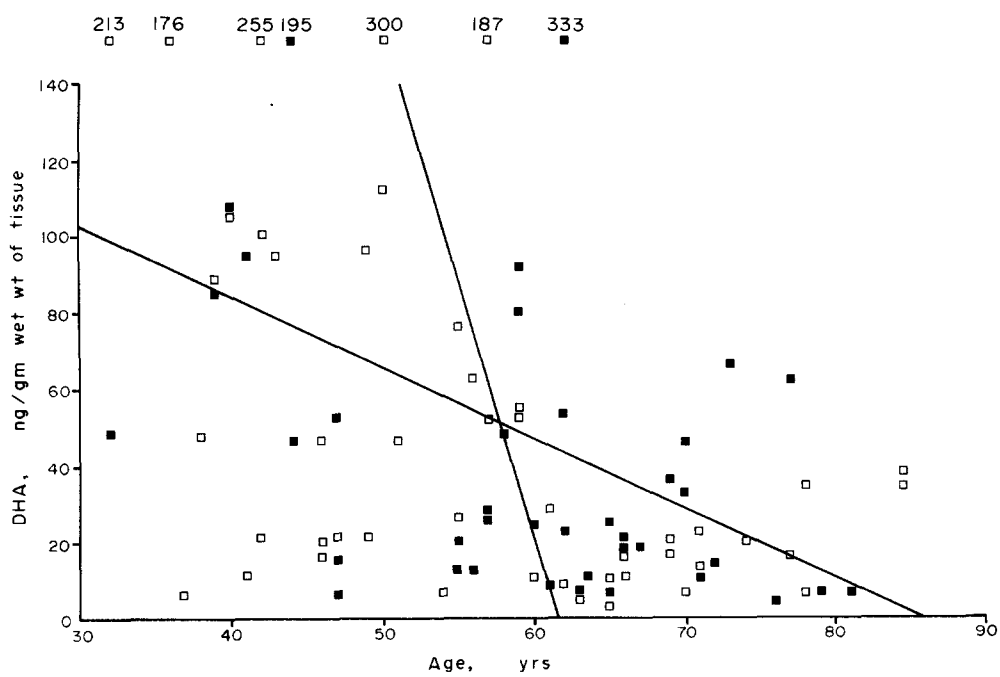

Fig. 1. DHA levels in human primary breast tumours. The lines are the regression lines, and the correlation between steroid levels and age ($R=0.38$) is significant ($P<0.001$). □ = oestradiol receptor negative and ■ = oestradiol receptor positive tumours.

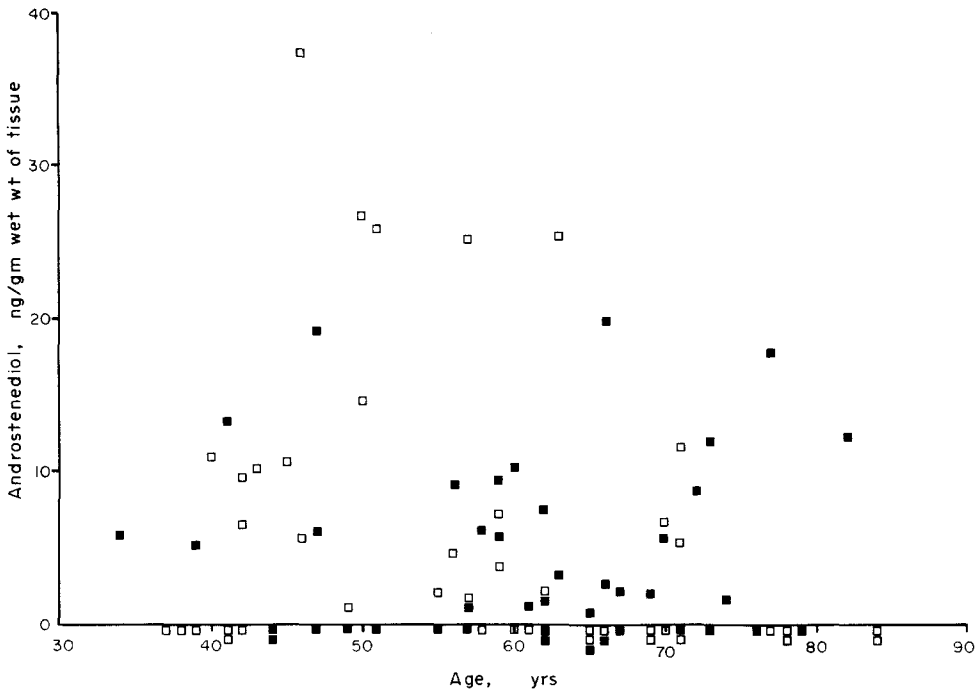

Fig. 2. Androstenediol levels in human primary breast tumours. □ = oestradiol receptor negative and ■ = oestradiol receptor positive tumours.

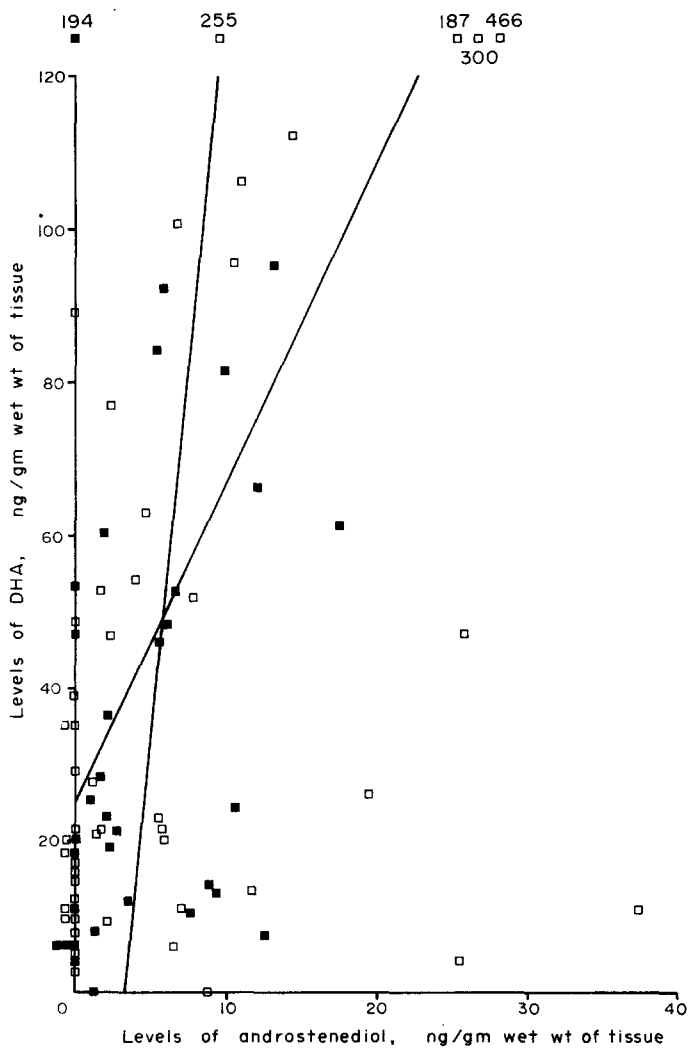

Fig. 3. Correlation between DHA and androstenediol content of primary breast tumours, both steroids measured in one extract. The two lines are the regression lines and the correlation between DHA and androstenediol levels ($R = 0.47$) is significant ($P < 0.001$). □ = oestradiol receptor negative and ■ = oestradiol receptor positive tumours.

5 fmole/mg cytosol protein) were found to contain high or low amounts of either steroid. Furthermore, tumours containing non-measurable amounts of androstenediol had the same distribution of receptor as those containing greater than the limit of the assay. However, tumours containing a high level of DHA (greater than 100 ng/g) were found to be mainly oestradiol receptor negative (7 out of 10 tissues), and similarly tumours with high levels of androstenediol (greater than 20 ng/g) also lacked measurable cytoplasmic receptor (5 out of 6 tissues). In the three tumours containing high levels of both steroids (Fig. 3) no specific cytoplasmic oestradiol binding could be detected.

DISCUSSION

All the tumours investigated contained measurable amounts of non-conjugated DHA at levels considerably greater than would be expected from contamination by plasma. Plasma DHA levels have been quoted [15] as being around 4.5 ng/ml in pre-menopausal women which drop to approximately 1.5 ng/ml after the menopause, whereas levels in these primary breast tumours were from 2.5 ng/gm to over 450.0 ng/gm. The correlation found between the level of this weak androgen in the tumour and the age of the patient might suggest that one possible source of the steroid may be the DHA sulphate found in high concentration in the plasma (over 2 μg/ml in pre-menopausal women and about 450 ng/ml post-menopause [15]). This would indicate that virtually all the breast tissues examined contained an active steroid sulphatase enzyme system, a concept which would agree with earlier work from these laboratories both *in vitro* [7] and *in vivo*, by perfusion [8], one of the principal metabolites of [^{3}H]DHA sulphate was shown to be the free steroid.

Dao and Libby [16] have suggested, however, that the level of steroid sulphotransferase activity correlates with response to endocrine therapy and more recently Godefroi *et al.* [17] have tentatively shown that there is a correlation between sulphotransferase activity and oestrogen receptor levels. The amounts of DHA found in these tumours may thus reflect a balance between the activities of sulphatase and sulphotransferase enzyme systems.

In the present series no correlation has been found between DHA levels in the tumour and the quantity of oestrogen receptor in the cytoplasm, with the exception that when amounts of endogenous steroid are high, tumours seem more likely to contain less available receptor sites. It is possible that if all the DHA is in the cytosol fraction there may be a direct effect on the oestrogen receptor assay although it has recently been shown [11] that DHA itself does not displace oestradiol from its receptor markedly even at very high concentrations.

If a relationship does exist between DHA in the tumour and physiological activity of the oestrogen receptor it would appear to be more likely that a metabolite of the C_{19}-steroid is responsible, possibly oestradiol itself or probably a C_{19}-steroid containing an hydroxyl group at the 3 and 17 positions. Many such steroids do displace oestradiol from its receptor [11], the most effective being androstenediol. Since this steroid is formed from DHA in both human [8] and rat [18] breast tumours, its concentration in the tissue may be very important, both in relation to the assay for the receptor, but more significantly, in its ability to modify the activity of the cell.

The correlation between DHA and androstenediol (Fig. 3) suggests that in many of the tumours there is an active 17β-hydroxysteroid dehydrogenase enzyme system. Although it has been reported that a similar conversion of oestrone to oestradiol and of androstenedione to testosterone occurs more frequently in neoplastic breast tissue than in normal tissue [19], it was of interest to find that in many tumours there were non-detectable levels of androstenediol. In these tumours it may be that this enzyme system is absent or that any androstenediol formed is rapidly metabolised to other compounds and does not accumulate within the tissue. In those tumours where androstenediol was found, however, the concentrations were again considerably greater than could be explained by reported plasma levels of this steroid [15].

Although it remains to be seen whether the DHA and androstenediol being measured are present in the cytoplasm of the cell or bound to particulate material or indeed merely stored in the fat particles in the tissue, the levels are, in many tumours, very high and may well be capable of affecting the oestrogen receptor and thus the activity of the cell. In some cases, these C_{19}-steroids in the primary tumour may act as a store of steroid precursor material from which oestrogens could be synthesised. It will be of particular interest to see whether the steroid content bears any relationship to the course of the disease or to the response to therapy of subsequent metastatic deposits.

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Biologic Characterization and Chemotherapeutic Response of MS-2 Tumor: a Non-Regressing Mouse Sarcoma Derived from MSV-M-Induced Sarcoma

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Abstract—*MS-2 tumor produced in BALB/c mice by transplant of* in vitro *cultivated cells derived from a primary MSV-M tumor in mice did not show spontaneous regression but grew progressively and killed the host. The MS-2 tumor was histologically classified as a malignant sarcoma. In syngeneic BALB/c mice, the i.m. transplanted MS-2 tumor had a mean doubling time of* 2.5 days *when the tumor mass was* 500 mg *and* 14 days *when the tumor reached* 4.0 g. *MS-2 tumor cells showed a high ability to metastatize (tested by bioassay). The organ mainly involved was the lung. In a small percentage of mice, metastases were also detected in spleen, liver, kidney, brain and blood. The activity of: cyclophosphamide, daunorubicin, adriamycin and 4′-epi-adriamycin on the MS-2 tumor was evaluated. The effects on both tumor growth and pulmonary metastases were detected. In parallel, the drugs were tested on the highly immunogenic T-MSV tumor, obtained by serial* in vivo *passages of the primary MSV-induced tumor. Daunorubicin did not have any therapeutic effect on the growth of MS-2 tumor; adriamycin produced a marked delay of the tumor growth and a significant increase of the life span; cyclophosphamide produced a longer delay in tumor growth than adriamycin did, but the life span was only slightly increased. On the T-MSV tumor, daunorubicin and adriamycin reduced tumor growth and did not affect spontaneous tumor regression; cyclophosphamide reduced tumor growth but completely abolished the spontaneous regression. Adriamycin and its new derivative 4′-epi-adriamycin, given to mice in which the MS-2 tumor had been surgically removed, inhibited the presence of tumor cells at the lung level.*

The MS-2 tumor studied in parallel with the T-MSV tumor allows the comparison of drug activity on a highly or weakly immunogenic tumor. Moreover, the MS-2 tumor is a viable model for studying the activity of chemotherapeutic drugs on lung metastases and their adjuvant effect on surgery.

INTRODUCTION

IN A RECENT report we have described a cell line, referred to as MS-2, established *in vitro* in our laboratory [1, 2] and derived from Murine Sarcoma Virus-Moloney (MSV-M)-induced tumors in BALB/c mice. Contrary to MSV-M-induced tumors, the tumor produced by injection of MS-2 cells into syngeneic hosts, called MS-2 tumor, did not show spontaneous regression, but grew progressively and killed the host [3]. The lack of spontaneous regression could be attributed to the low antigenicity of MS-2 cells due to the low sarcoma virus release: in mice with growing MS-2 tumor no concomitant immunity was detectable [3]. The MS-2 tumor can be used as a new experimental model for investigations on the activity of antineoplastic drugs. Parallel experiments on the MS-2 tumor and on the spontaneously regressing MSV-M tumor can supply information on the ratio between antitumor and immunodepressive activity of antineoplastic drugs.

In the present report, the metastatic dissemination and other biological properties of MS-2 tumor are described, as well as the chemotherapeutic activity of cyclophosphamide, daunorubicin, adriamycin and 4′-epi-adriamycin, a recently synthesized analog of adriamycin [4, 5].

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MATERIAL AND METHODS

Animals

The animals used throughout these experiments were 6–8 weeks-old syngeneic BALB/c, DBA/2, C3H, C57BL/6 mice and hybrid BDF_1 (C57Bl × DBA/2) mice, obtained from Charles River Breeding laboratories, Calco, Italy.

Tumors

MS-2 tumor was maintained by serial intramuscular (i.m.) passages of a tumor cell homogenate into the right hind leg. The experiments were performed on mice with tumors at the 36–46th generations. In the studies on the growth characteristics of the MS-2 tumor, cell dispersion was accomplished by Reinhold's method which was slightly modified [6]. A Moloney transplanted sarcoma (T-MSV-M) was originally induced in BALB/c mice by i.m. infection with MSV-M. This tumor has been maintained by serial i.m. passages in syngeneic hosts for more than 2 yr [3]. Tumor growth was followed by caliper measurement of two perpendicular diameters and tumor weight was estimated according to Geran *et al.* [7].

Oncogenic dose 50 (OD_{50})

Serial dilutions of viable cells were inoculated i.m. into syngeneic mice. The OD_{50} was derived graphically from a plot relating the size of the inoculum to the number of mice developing tumors within 120 days from the day of tumor inoculation.

Assays for metastasis detection

Lung, spleen, liver, kidney and brain from mice bearing MS-2 tumor were tested for the presence of tumor cells by bioassay. Blood metastases were detected by i.m. or i.v. injection of 0.2 ml/mouse of heparinized blood from tumor-bearing mice. The tumor-bearing mice were killed by cervical dislocation at intervals of 5–10 days after i.m. transplant of 10^6 tumor cells. The organs were removed aseptically, minced, inserted into a syringe with a small amount of 199 medium, and injected s.c. into the right inguinal region of normal BALB/c mice.

The organs from each animal were bioassayed individually, except for liver. The whole liver from each mouse was carefully minced and divided into two equal fractions. Each fraction was then implanted s.c. into the right inguinal region of a recipient mouse. The tumors grown after s.c. implant of organs tested for the presence of metastatic cells were always examined histologically. Mice were observed for 100–120 days after tissue implantation.

In some experiments, pulmonary metastases were also evaluated by lung perfusion with a suspension of India ink (15% v/v in water) followed by fixation in Fekete's solution [8].

Surgical tumor removal

One million MS-2 sarcoma cells were injected into a footpad; surgical amputation of tumor-bearing legs was performed on animals anesthetized with Farmotal (Farmaceutici Italia). A skin incision was made all around the upper thigh as far as possible above the area of tumor growth. The muscle and femur were cut with steril scissors. the wound was closed by clamping with metal wound clips. Surgical removal resulted in less than 10% surgical mortality.

Chemotherapeutic assays

BALB/c mice were inoculated with MS-2 or T-MSV-M tumor cells and treated i.v. or i.p. (Cyclophosphamide was received from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Daunorubicin, adriamycin hydrochloride, and 4′-epi-adriamycin were supplied by Farmitalia, Milan, Italy. The drugs were dissolved in Ringer solution immediately before use and injected at equitoxic doses in a vol of 10 ml/kg of body weight.

Histological examination

The tumor, lung and liver samples for microscopic examination were fixed in 10% formaldehyde or Bouin's solution, embedded in paraffin, and stained with hematoxylin and eosin. One portion of tumor was fixed in Karnowsky's solution and embedded in epoxy–Epon resin (0.75 nm thick). Semithin sections were stained with methylene blue and Azur II. Gomori's silver impregnation method was used for reticulum fibers.

RESULTS

Growth characteristics of MS-2 tumor

Table 1 shows the results obtained in mice injected with graded numbers of MS-2 tumor cells. The OD_{50} was 4.4×10^2 cells/mouse.

Table 2 presents data from nine experiments carried out in mice injected with 10^4 MS-2 cells/mouse. On 217 injected mice we observed 100% tumor takes: the average of tumor insurgence was 14.2 days and no spontaneous regressions and no survivors were observed. The median day of death from 9 experiments was day 42.8, with a range of medians in single experiments of 38–53 days and a range of deaths in individual mice of 19–76 days.

In Fig. 1 the growth curve for MS-2 tumor is

Table 1. Oncogenicity of MS-2 tumor cells implanted i.m. in adult BALB/c mice

No. cells/mouse	No. of tumor takes/total*	%
10^4	30/30	100
10^3	24/30	80
5×10^2	18/30	60
10^2	6/30	20
5×10	2/30	6.6

*Mice observed for 100 days.

Table 2. Life span of BALB/c mice implanted i.m. with 10^4 MS-2 cells/mouse

Exp. No.	Average time of tumor insurgence (days)	Median life span (days)	Range of deaths (days)	No. of survivors/ total
MC-31	17.3	38.5	27–54	0/10
MC-34	17.0	40.0	35–62	0/10
MC-35	13.8	47.0	33–56	0/10
MC-36	16.7	40.5	29–55	0/10
MC-39	13.1	40.0	29–47	0/10
MS-98	12.0	41.0	27–69	0/57
MS-102	12.0	46.0	19–54	0/91
MS-108	12.1	39.0	32–50	0/9
MS-112	14.3	53.5	47–76	0/10
Average of all experiments	14.2	42.8	19–76	0/217

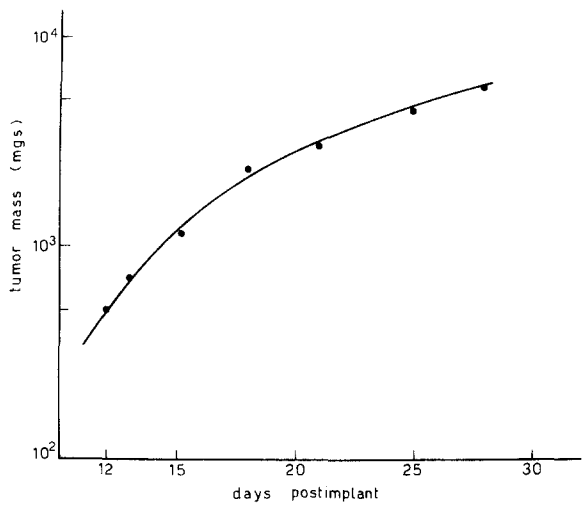

Fig. 1. Gompertz fitted growth curve for MS-2 tumor implanted i.m. into adult BALB/c mice (10^6 cells/mouse). Mean of 224 animals.

shown. The growth of i.m. injected MS-2 tumor is well described by a Gompertz function, and the doubling time increases from 2.5 days for a 0.5 g tumor (500 mg) to 3 days for a 1.0 g tumor, and reaches 14 days when the tumor is 4.0 g.

The i.m. transplant of MS-2 tumors in syngeneic, allogeneic and hybrid mice was tested. Results summarized in Table 3 show that progressive tumor growth was obtained only in syngeneic BALB/c mice, in DBA/2 mice bearing the same H-2 locus allele, or in BDF_1 (BALB/c × DBA/2) mice. In allogeneic C3H and C57 BL/6 mice, the MS-2 tumor did not grow.

Table 3. Effect of i.m. transplant of MS-2 tumor cells in syngeneic and allogeneic mice

Mouse strain	Allele of H-2 locus	Growth of MS-2 tumor
BALB/c	d	progressive
DBA/2	d	progressive
BDF_1 (C57Bl × DBA/2)	d	progressive
C3H	k	absent
C57BL/6	b	small tumors which regress

Histological characteristics

The general histological features of the MS-2 tumor 16 days after MS-2 cell injection have been previously described [2]. Microscopic examination of the tumor revealed that there

was little or no evidence of the establishment of a capsule that would form any sort of barrier to extension of tumor growth. The s.c. connective tissue in the region of the spreading tumor showed edema, sparse inflammatory cell infiltration and hyperemic blood vessels (Fig. 2). At the periphery of the tumor, where the cells were loosely arranged, they appeared rounded or slightly elongated (Fig. 3). The cytoplasm was basophilic and homogeneous and the nuclei were round or oval and hypercromatic. The nucleous membrane enclosed delicate or dense chromatin granules and one or more large nucleoli (Fig. 4). Many mitotic features were observed. The tumor had numerous reticular fibers (Fig. 5) intimately associated with the cells. On the basis of these features, the tumor was classified as a malignant sarcoma.

Metastases

The ability of i.m. transplanted MS-2 tumor to metastatize was tested by bioassay of lung, liver, spleen, kidney, brain and blood of MS-2 tumor-bearing mice at different times after tumor transplant. The results are summarized in Table 4. Five days after tumor transplant no tumor cell was present in the tested samples except in liver. At 10 days, the incidence of lung metastases was already 100% in mice bearing a palpable tumor. Spleen and kidney metastases were detected the 20th day after tumor implant in a small percentage of mice. Brain metastases were present only at a very late stage of the disease. In liver, tumor cells were detected only occasionally. In blood, tumor cells were detectable only 30 or 40 days after the tumor transplant in one or five mice tested; i.v. injection of blood from tumor-bearing mice produced similar results.

The histological features of lung and liver metastases are shown in Figs. 6 and 7. The metastatic cells appeared rounded with hyperchromatic nuclei. In lungs, gross metastatic nodules were observed, with tumor cells invading the large vessels (Fig. 6). In liver, grapes of 2–3 tumor cells were observed in the capillary lumen (Fig. 7). All tumors were classified as malignant sarcomas.

Lung metastases were also evaluated by gross examination of lungs after perfusion with a suspension of India ink. Marked differences existed between the two evaluations. As expected, the Wexler's method was definitely less sensitive than the bioassay.

The histological features of tumor derived from s.c. implant of lung and liver are similar to those of MS-2 tumor.

The lung of BALB/c mice bearing footpad tumors were bioassayed 8, 10, 13, 16 and 20 days after tumor implant. The results reported in Table 5 show that when surgical removal was performed 8 or 10 days after tumor implant, 53 or 66%, respectively, of mice had developed lung metastases. When surgical removal of the tumor was performed 13 days after tumor implant, 100% of the mice had developed metastases.

Response to drug treatment: activity on primary tumor and on lung metastases

The effects of daunorubicin, adriamycin and cyclophosphamide on the growth of MS-2 and T-MSV-M tumors, tested in parallel in BALB/c mice, are shown in Figs. 8 and 9 and Table 6. The drugs were administered i.v. or i.p. 6, 10, 14 and 18 days after the i.m. tumor implant. In MS-2 tumor-bearing mice (Fig. 8 and Table 6)

Table 4. Organ bioassay of mice bearing i.m. implanted MS-2 tumor

Donor mice		Recipient mice					
Days after tumor implant	Average weight of primary tumor (g)	No. of animals with tumors*/No. of mice inoculated with					
		Lung	Spleen	Kidney	Brain	Liver†	Blood
5	no tumor	0/5	0/5	0/5	0/5	2/10	0/5
10	no tumor	0/5	ND‡	ND	ND	ND	ND
	palpable tumor	10/10	0/10	0/10	0/5	2/10	0/5
15	0.77	15/15	0/10	0/10	0/5	2/10	0/5
20	1.78	15/15	1/15	3/15	0/5	0/5	0/5
25	3.25	9/10	0/10	4/10	1/5	2/10	1/5
30	4.60	10/10	2/10	4/10	0/5	0/5	1/5
40	8.20	5/5	1/5	0/5	1/5	0/5	1/5

*Tumors in recipient mice were assessed histologically.
†The liver from a tumor-bearing mouse was divided into equal fractions and each one was implanted i.m. into recipient mice.
‡Not done.

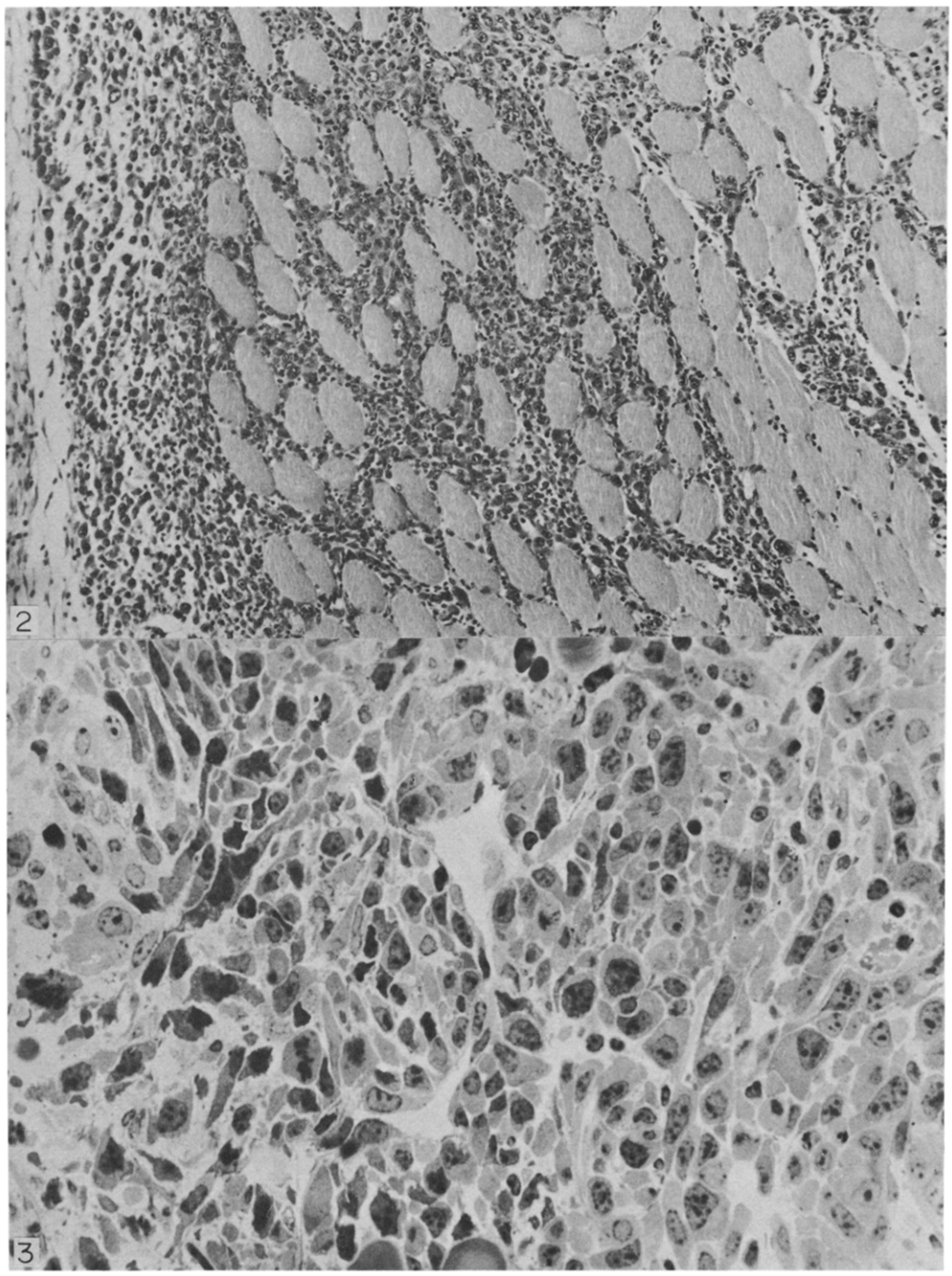

Fig. 2. Photomicrograph of MS-2 tumor at the 15th day *after the i.m. implant in BALB/c mice. Hematoxylin and eosin,* × 100.

Fig. 3. Photomicrograph of MS-2 tumor at the 20th day *after i.m. implant in BALB/c mice. Epon-Araldite sections* (0.75 nm *thick*), *methylene blue and Azur* II, × 400.

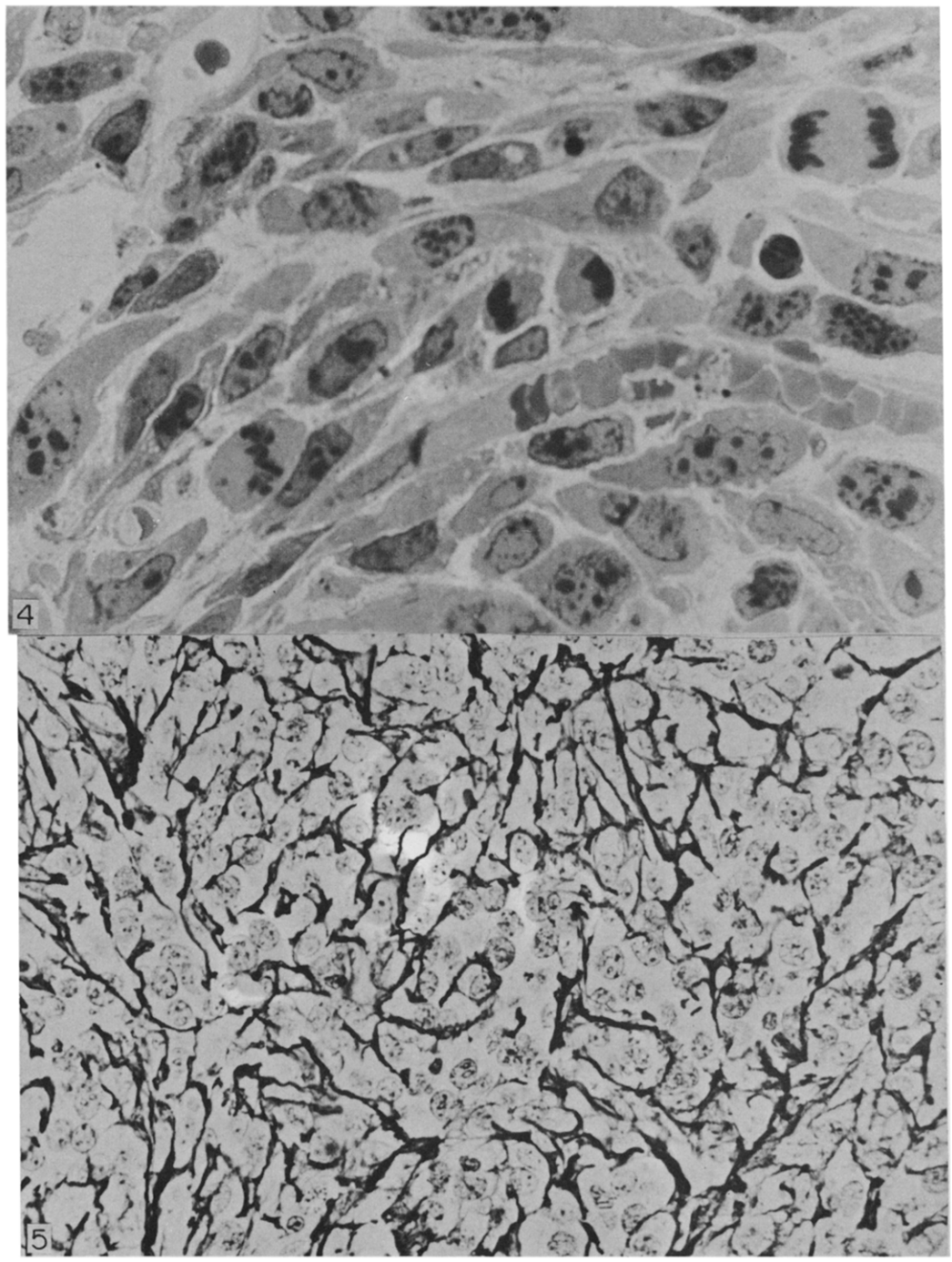

Fig. 4. *Photomicrograph of MS-2 tumor at the* 20th day *after i.m. implant in BALB/c mice. Epon-Araldite sections, methylene blue and Azur II,* × 1000.

Fig. 5. *Photomicrograph of MS-2 tumor at the* 20th day *after i.m. implant in BALB/c mice. Gomori's silver impregnation,* × 400.

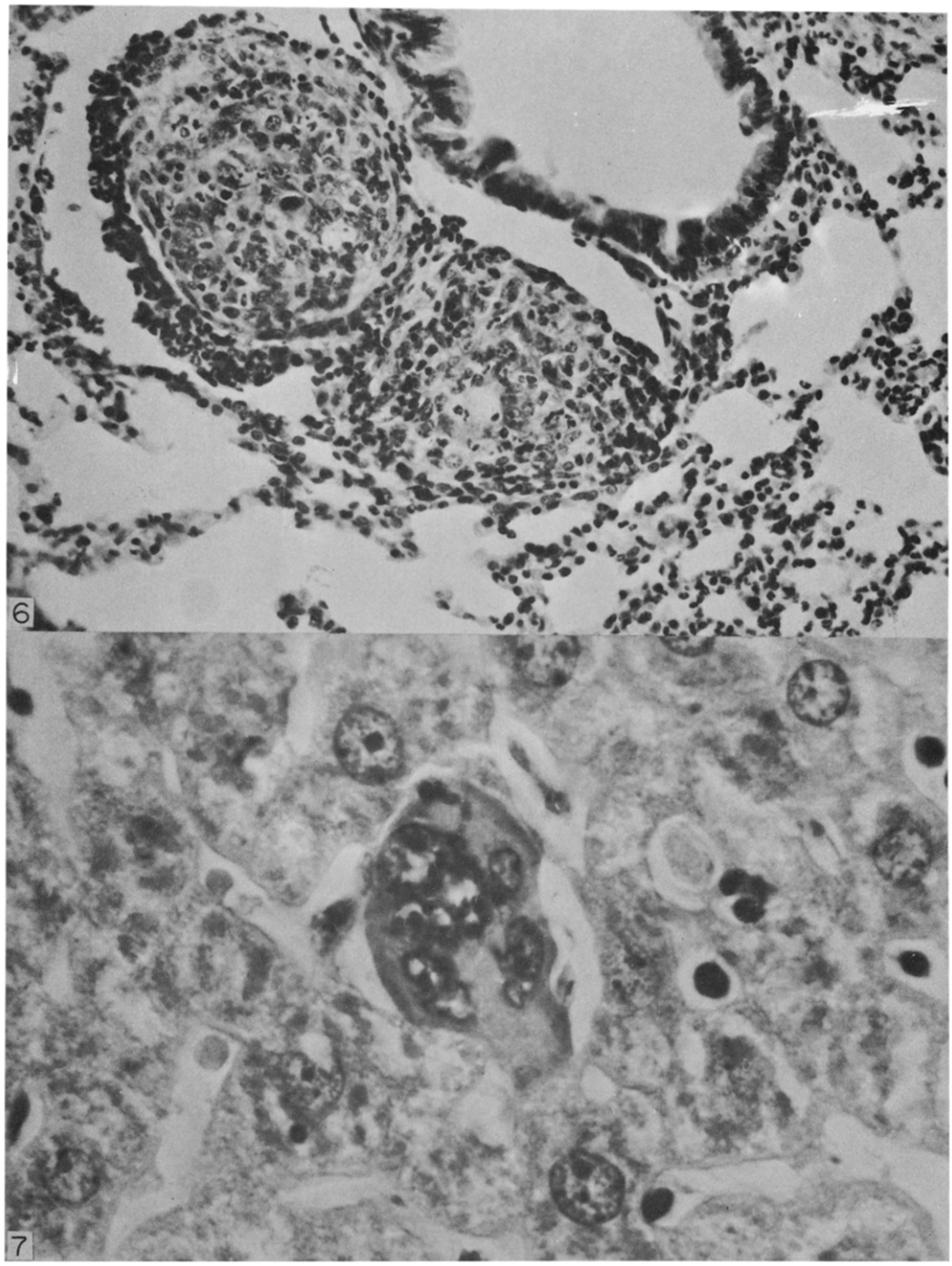

Fig. 6. *Photomicrograph of lung from a BALB/c mouse bearing MS-2 tumor at the* 20th day *after i.m. tumor implant. Hematoxylin and eosin,* × 250.

Fig. 7. *Photomicrograph of liver from BALB/c mouse bearing MS-2 tumor, at the* 25th day *after i.m. tumor implant. Hematoxylin and eosin,* × 1000.

Table 5. Pulmonary metastases in BALB/c mice at different times after footpad implant of MS-2 cells*

Days after tumor implant into donor mice	Recipient mice	
	Mice with tumor/total	Incidence of metastases %
8	8/15	53
10	8/12	66
13	13/13	100
16	8/9	100
20	8/8	100

*Detected by bioassay of donors lungs.

treated according to this schedule, daunorubicin did not show any therapeutic effect. Adriamycin, administered i.v. at the dose of 3.5 mg/kg/day, produced a delay of tumor growth (Fig. 8) and a significant increase of the life span ($P<0.01$; $T/C\% = 145$) (Table 6). Cyclophosphamide produced a higher delay in tumor growth but a slightly lower life span increase at the highest doses tested (50 mg/kg/day i.v. and 60 mg/kg/day i.p.; $P<0.05$).

On T-MSV-M tumor, treatment with adriamycin or daunorubicin (Fig. 9) resulted in a slight inhibition of tumor growth followed by tumor regression which was more rapid than in controls. The treatment with cyclophosphamide resulted in inhibition of tumor regression: most mice died with progressively growing tumors.

The effect of adriamycin and 4'-epi-adriamycin on lung metastases is shown in Table 7. The experiment was carried out on MS-2 tumor-bearing mice in which the tumor was removed 10 days after implant. The mice were treated i.v. with the two drugs 2, 9, 16 and 23 days after tumor removal. The lung bioassay was carried out 3 days after the last treatment. At the lowest and highest doses tested (8 and 12mg/kg/day), adriamycin, administered according to this schedule of treatment did not inhibit the appearance of lung metastases. At the intermediate dose tested (10 mg/kg/day), the appearance of lung metastases was slightly inhibited 4'-epi-adriamycin was more active than adriamycin in reducing the percentage of lung metastases. In mice treated with 4'-epi-adriamycin, at the intermediate dose (10mg/kg/day), no tumor cells were detected by the bioassay at the lung level.

DISCUSSION

Model systems involving transplantable tumors have been used extensively in the screening and evaluation of antitumor agents and the value of such systems in the selection of compounds with activity in clinical neoplasia has been documented [9]. The MS-2 tumor used in this study possesses several biological characteristics desirable in a chemotherapeutic model for human cancer, the majority of which are slow growing tumors. The MS-2 tumor has the

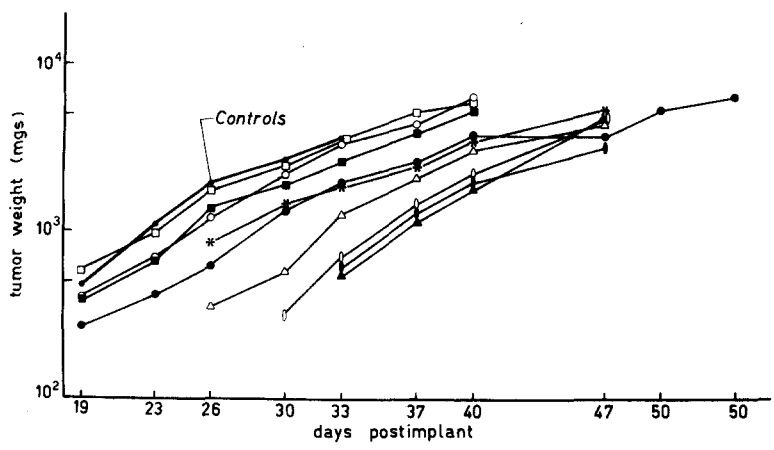

Fig. 8. Effect of adriamycin, daunorubicin and cyclophosphamide, on i.m. implanted MS-2 tumor growth in BALB/c mice.

Treatment at days 6, 10, 14 *and* 18 *after tumor implant.* (○), *adriamycin*, 2.5mg/kg/day, *e.v.*; (●), *adriamycin*, 3.5mg/kg/day, *e.v.*; (□) *daunomycin*, 3.25mg/kg/day, *e.v.*; (■) *daunomycin*, 4mg/kg/day, *e.v.*; *cyclophosphamide*; (*), *CY*, 20mg/kg/day, *e.v.*; (0), *CY*, 40mg/kg/day, *e.v.*; (⧫), *CY*, 50mg/kg/day, *e.v.*; (△), *CY*, 40mg/kg/day, *i.p.*; (▲), *CY*, 60mg/kg/day, *i.p.*

On the abscissa, days postimplant; on the ordinate, tumor weight (mg).

Table 6. Activity of adriamycin, daunorubicin and cyclophosphamide on MS-2 tumor

Compound	Treatment* Route	Dose (mg/kg/day)	MST†	T/C‡ %
—	—	—	45.5	—
Adriamycin	i.v.	2.5	57.5	126
		3.5	66.0§	145
Daunorubicin	i.v.	3.25	48.5	107
		4.0	51.0	112
Cyclophosphamide	i.v.	20	57.5	126
		40	57.0	125
		50	61.0¶	134
Cyclophosphamide	i.p.	40	54.5	120
		60	60.5¶	133

*Compounds were administered at days 6, 10, 14 and 18 after tumor implant.
†Median survival time (days).
‡Median survival time of treated mice/median survival time of control mice × 100.
§$P<0.01$ as evaluated by Student 't' test.
¶$P<0.05$ as evaluated by Student 't' test.

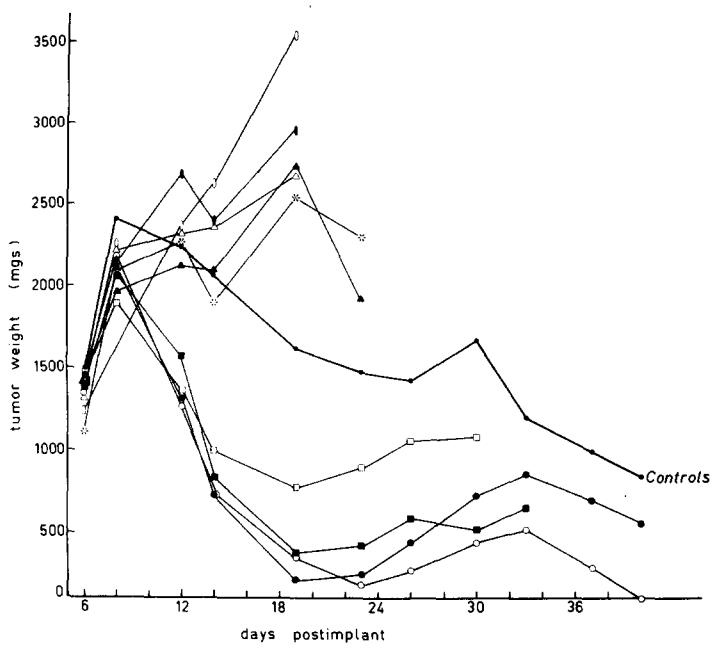

Fig. 9. Effect of adriamycin, daunorubicin and cyclophosphamide on i.m. implanted T-MSV-M tumor. See legend to Fig. 8.

histologic features of malignant sarcoma, i.e. high oncogenicity, a slow growth rate, low antigenicity [3] and rapid metastatization and these characteristics were maintained after thawing. The MS-2 tumor, similarly to the Ki-MSV non producer tumor described by Ikawa *et al.* [10], is derived from cells infected with MSV-M; but it differs from the MSV-M-induced tumor in several characteristics, mainly in its lack of spontaneous regression. The MS-2 tumor differs from the Ki-MSV non-producer tumor in that it has been obtained from cells infected *in vivo* with MSV-M and because it has been histologically classified as a sarcoma, whereas the Ki-MSV non-producer tumor was classified as an epithelial tumor [10].

In a previous paper [3] we have reported that the MS-2 tumor was not sensitive to treatment with daunorubicin and adriamycin. The experiments reported here show that, with the schedule of treatments applied, adriamycin and cyclophosphamide, but not daunorubicin, were able

Table 7. Activity of adriamycin and 4′-epi-adriamycin on MS-2 lung metastases

Compound*	Dose (mg/kg/day)	Bioassay of lungs: No. of mice with tumor/total	%
—	—	5/7	71
Adriamycin	8	4/7	57
	10	2/7	28
	12	4/7	57
4′-*epi*-adriamycin	8	2/7	28
	10	0/7	0
	12	3/7	42

*Compounds were administered i.v. at days 2, 7, 16 and 23 after surgical removal of MS-2 tumor, which was carried out at the 11th day after MS-2 tumor cell implant into the footpad.

to inhibit the MS-2 tumor growth. Cyclophosphamide was more effective than adriamycin in delaying the tumor growth, but adriamycin was slightly more active than cyclophosphamide in increasing the life-time of the tumor-bearing mice.

An explanation for these findings was afforded by the data obtained on the highly immunogenic, spontaneously regressing T-MSV-M tumor. It was observed that adriamycin did not hamper tumor regression, but cyclophosphamide strongly inhibited this process. Therefore, it can be supposed that, although cyclophosphamide produced an inhibitory effect on the primary MS-2 tumor, it was probably ineffective in curing pulmonary metastases. In fact, the development of metastases is influenced by the immunologic competence of the host [11, 12] and the immunologic impairment has been shown to have a more striking effect on metastatic growth and development than on primary tumor growth [13]. Moreover, in a recent report Carmel and Brown [14] found a significant enhancement of lung colony formation by pretreatment of mice with cyclophosphamide.

The data reported here show that adriamycin and its new derivative 4′-epi-adriamycin given to mice in which the MS-2 tumor has been surgically removed inhibit the presence of tumor cells at the pulmonary level. The optimal dose was, for both compounds, 10 mg/kg/day; at higher doses both compounds were less active, probably because of the immunodepressive effect [15, 16].

In the experiments carried out by surgical removal of i.m. implanted tumors, an undesired complication is often present due to local recurrence of the primary tumor, which can hamper the validity of the final results [17]. We have overcome this by implanting the MS-2 tumor cells into the foot-pad: surgical removal of the primary tumor is facilitated and local recurrence of primary tumor is minimized.

The availability of a new model system which allows the detection of antitumor activity, effect on metastases, immunodepressive activity (even if indirectly by comparison with data obtained in other models), can be of usefulness for chemotherapeutic studies. In addition, the MS-2 tumor offers the possibility to study the *in vitro* activity of antitumor agents [1]. These data confirm that results of chemotherapeutic activity on MS-2 and T-MSV-M tumors can be of great help for a better understanding of the properties of antitumor drugs.

The MS-2 tumor, besides allowing the comparison of drug activity on highly or weakly immunogenic tumors, is a viable model for studying the activity of chemotherapeutic drugs on lung metastases and their adjuvant effect on surgery.

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The Effects of Prednisolone on Normal Pigs*

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Abstract—*Prednisolone at doses of* 0, 2, 4 *and* 6 mg/kg *was given daily* 5 *days/week for* $5\frac{1}{2}$ *weeks to* 4 *pairs of normal Large White sibling pigs. One of each pair was then killed. Treatment caused a dose-related reduction in the size of the thymus with "overshoot" on recovery. A similar but less spectacular reduction, without "overshoot", on recovery, occurred in the lymph nodes and blood lymphocyte counts. There was also a reduction in serum total globulin levels and a complementary increase in serum albumin during treatment. Treated pigs showed a reduced contact sensitivity to DNFB during the last week of treatment. No changes attributable to treatment were observed in blood neutrophil and platelet counts nor in the red cell pictures.*

It is concluded that the pig falls in the "corticosteroid-resistant" group of species which includes man.

INTRODUCTION

THE EXISTENCE of a hereditary form of lymphoma in Large White Pigs was first reported by McTaggart *et al.* [1]. The clinical, haematological and pathological features of 53 cases were described by Head *et al.* [2]. The condition has been shown to be transmitted in association with an autosomal recessive gene. Diagnosis may be made as early as 4 weeks of age but more commonly at 2–3 months. Clinical features include marked stunting of growth, lymphadenopathy, muscle wasting and the development of a pot belly. No affected pig has yet reached functional sexual maturity (normally reached at about 6 months) despite the fact they may live to the age of 15 months. As the disease is now known to be predictable in its course, it seemed appropriate to use the herd for evaluation of new cytotoxic drugs. The tumour has marked similarities to poorly differentiated lymphocytic lymphoma in man and may prove a more predictable system than established small laboratory animal lymphomas. The suitability of the pig as a model was examined by testing with a selection of drugs already in common use.

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Reprints from H. S. McTaggart, Department of Veterinary Medicine, Easter Bush, Roslin, Midlothian, Great Britain.

Prednisolone was chosen as the first drug for assessment as it is known in several experimental models to be a useful cytolytic agent for lymphocytes [3]. It induces a limited temporary remission in acute lymphatic leukaemia in children, although it has a much enhanced effect when combined with vincristine. It also has beneficial effects in B lymphocyte tumours such as some of the non-Hodgkin lymphomas, myeloma and macroglobulinaemia [4].

This paper will describe the methods used in toxicity testing with particular reference to the monitoring of the effects of different levels of prednisolone on non-lymphomatous Large White pigs. The effects of prednisolone on pig lymphoma cases and of other steroids and cytotoxic drugs will be reported later.

MATERIAL AND METHODS

Animals

A litter of 8 normal Large White pigs (5 females, 3 castrated males) was used. These animals were of the lymphoma strain but were of non-carrier parentage. They were weaned at 8 weeks and housed in the same pen. Monitoring was commenced 2 weeks later. Animals were fed on "Breeder's Cubes" (SAI Ltd.) with water *ad libitum*. At weekly intervals from 3 weeks before treatment commenced the animals were weighed and blood samples were taken from the anterior vena cava [5]. An EDTA sample was taken for

routine haematology and a clotted sample for calcium, magnesium, inorganic phosphate, sodium, potassium, chloride, total protein and protein fraction estimations. Animals were also sensitised with dinitro-fluorobenzene (DNFB) so that recall to this antigen could be tested at later periods throughout treatment. Four groups of 2 pigs selected by lot were treated with 2, 4 or 6 mg/kg of prednisolone sodium phosphate solution (Codelsol, MSD, 16 mg/ml) or with normal saline corresponding to the volume of the 4 mg/kg injection. The dose was given i/m daily for 5 days each week. Clinical observations were undertaken each day. After $5\frac{1}{2}$ weeks of treatment a bone marrow core biopsy was taken from the crest of the ileum, using an 11 gauge Jamshidi needle (Kormed Inc.). One pig from each pair was then killed by intravenous pentobarbitone overdose and an immediate autopsy carried out. Treatment of the remaining animals continued during the week, being reduced to half, quarter and one-eighth during the last 3 days before being finally discontinued. Five weeks later, when it was considered that the visible effects of the drug had disappeared, a bone marrow core biopsy was taken and the animals were slaughtered.

Haematology

Red and white cells were counted using an electronic counter (Coulter Electronics Ltd., Model ZF). Platelets were counted using a white cell pipette, an Improved Neubauer haemocytometer and Baar's diluting fluid without brilliant cresyl blue. White cell differential counts were based on 200 cells counted in Leishman-stained smears. Haemoglobin was estimated by the cyanmethaemoglobin method using a Coulter haemoglobinometer, and packed cell volume (PCV) using a Gelman Hawksley microhaematocrit centrifuge. Mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and mean cell haemoglobin (MCH) were obtained by calculation.

Biochemistry

Serum sodium and potassium were measured by flame emission and calcium and magnesium by atomic absorption spectrophotometry (Shandon Southern, Model A3400). Serum chloride was measured in an EEL chloride meter (Evans Electroselenium Ltd.) and inorganic phosphate by the method of Fiske and Subbarow [6]. Total protein was estimated by the biuret method of Henry *et al.* [7] and albumin by the bromcresol green dye-binding method described by Dumas *et al.* [8]. On selected occasions a more detailed analysis of protein fractions was carried out by electrophoresis on Millipore cellulose acetate strip (Phoroslide) at 100 V for 20 min. Proteins were stained with Ponceau S, decolourized with 5% acetic acid, dehydrated in ethyl alcohol for 5 min, cleared in acetic acid-ethyl acetate for 1 min and dried in a warm oven. Protein fraction readings and a permanent trace were obtained for each sample using a Millipore Phoroscope densitometer.

Cell-mediated response

Delayed hypersensitivity reactions were measured by contact sensitivity to DNFB in equal volumes of acetone and dimethylsulphoxide (DMSO) [9]. An area of skin between the shoulder blades or the iliac crests was prepared by removing the hair with scissors and cleaning with spirit. On each occasion 5 μl DNFB solution was applied with an automatic micropipette and spread within an area of 14 mm in diameter with the flattened end of a glass rod. Pigs were sensitized with 10% DNFB. A preliminary test 20 days later with 1% DNFB indicated that this concentration was too weak (Table 1). Subsequent tests were carried out during and after the course of prednisolone treatment, using 5 or 10% DNFB, readings being made at 24 hr and, sometimes, at 48 hr. An interval of at least 7 days was allowed to elapse between tests.

Pathology

The autopsy examination was started immediately after an animal had been killed so that the tissues selected for fixation were placed in buffered neutral formol saline within a few minutes and never more than 20 min after death. These tissues were processed to paraffin wax, sectioned at 6 μm and stained with haematoxylin and eosin. Certain sections were also stained with Gordon and Sweet's stain for reticulin fibres, Martius scarlet blue, periodic acid-Schiff reaction, Perls' prussian blue method for haemosiderin and Leishman's stain.

In the course of the autopsy the lymph nodes throughout the body, thymus, spleen, kidney, liver and adrenal glands were dissected out and weighed. In the case of bilateral organs, mean values were obtained for kidneys and adrenals but only one of a pair of lymph nodes, always from the same side, was weighed. The absolute weights were then expressed as relative weights in centigrams per kilogram of body weight. The relative

Table 1. *Effect of prednisolone on skin reactions of individual pigs to dinitrofluorobenzene (DNFB)*

Days from primary sensitization	0	20	27	35	42	43	67	68	81	82
% DNFB applied	10	1	10	10	5		5		10	
Prednisolone dose—Control	0	0	2	4	4	4	–	–	–	–
	4	0	3	3	3	3	0	0	3	4
2 mg	3	0	3	3	0	0	–	–	–	–
	3	0	2	3	1	3	2	1	3	4
4 mg	0	0	1	3	1	1	–	–	–	–
	4	0	3	3	3	2	0	0	4	4
6 mg	1	0	2	2	0	0	–	–	–	–
	3	0	3	3	0	1	1	0	3	4

0, negative; 1, slight erythema; 2, erythema and swelling; 3, small ulcerations; 4, large ulcerations. Treatment with prednisolone commenced on the 11th day and ceased or was tailed off from the 44th day after sensitization.

weights of the "bronchial", "head" and "iliac" groups of nodes represent the totals for the various nodes in each of these regions.

The biopsy core samples of bone marrow were initially fixed in buffered neutral formol saline and then post-fixed in Bouin's fluid for 48 hr and decalcified in EDTA. In addition, the bone marrow of the body of the first lumbar vertebra and the wing of the ileum including the crest were taken as 5 mm slices cut with a saw and decalcified in formic formol before processing to paraffin wax. These latter samples were used to assess the value of the biopsy method.

To facilitate comparison between samples during the histological examination groups of sections were ranked in degree of change using a split-field comparator microscope. Where a structure could be drawn in outline (e.g. liver lobule, hepatic cell, white pulp pattern of spleen or thymic lobule), a microscope fitted with a Wild drawing tube was employed to demonstrate the degree of change present.

RESULTS

Clinical observations

Throughout the experiment, all pigs were bright and active. Prednisolone did not appear to depress interest in food but as individual intakes could not be measured the effect on appetite could not be assessed. Rectal temperatures fluctuated from day to day within the normal range for pigs of this age. All pigs on prednisolone, irrespective of dose, were noted to have marked polydipsia and polyuria. At the higher dose levels, they became pot-bellied. After 3 weeks of treatment, the 2 pigs receiving 4 mg/kg doses showed rectal eversion but this corrected itself spontaneously. The mean weight gain during the 5 weeks of treatment (Table 2) was lower in the 6 mg dose group (5.5 kg) than in any other group (controls, 8.5 kg; 2 mg dose, 7.5 kg; 4 mg dose, 9.0 kg). Similarly, during the post-treatment period, weight gain was lowest in the 6 mg dosed pig (14.5 kg as against 18.5, 18.0 and 19.0 kg respectively), despite the fact that its commencing weight was average for the litter.

Haematology

There was a steady decline in mean absolute lymphocyte counts in all groups during the treatment period. This decline was dose-related, being least in the controls and greatest in the 6 mg dosed group (Table 3, $r=0.75$, df=6, $P<0.05$, >0.02; $y=1835+564\ x$). The percentage drops in lymphocyte counts in the 4 groups were 5.8, 23.8, 31.5 and 42.6 respectively. During the posttreatment period the absolute lymphocyte counts remained more or less static.

Eosinophil counts showed a mean decrease in the control group during the treatment period whereas the treated groups all showed increases (Table 3).

Neutrophil counts showed a mean decrease in all groups during the treatment period (Table 3) but, as in the case of platelet counts and red cell pictures, there were no differences between groups which could be attributed to the treatments.

Biochemistry

Observations based on electrophoresis indicated that serum albumin levels increased in all groups during the treatment period (Table 4). This increase was exaggerated in

Table 2. Effect of prednisolone on body weight (kg) *and on relative weights of organs* (cg/kg *body weight*) *of individual pigs*

	Pigs killed at end of treatment				Pigs killed at end of experiment			
Prednisolone dose	Control	2 mg	4 mg	6 mg	Control	2 mg	4 mg	6 mg
Body weight:								
Before treatment	15	19.5	21	19	20.5	14.5	23	19
At end of treatment	22	27	29	23.5	30.5	22	33	25.5
At end of experiment	—	—	—	—	49	40	52	40
Lymph nodes:								
Mesenteric and colonic	523	341	324	374	408	325	250	275
Gastric and splenic	33	28	25	24	31	34	32	32
Bronchial group	11	13	9	8	10	8	7	11
Total splanchnic	567	382	358	406	449	367	289	318
Head group	30	27	30	24	24	28	21	21
Iliac group	8	10	6	7	8	10	8	6
Prescapular	3	2	7	4	3	5	3	5
Precrural	4	3	3	2	2	3	2	3
Inguinal	15	12	13	9	12	10	10	10
Popliteal	2	2	1	0.4	N.F.	1	1	1
Total superficial	62	56	60	46	49	57	45	46
Thymus	91	69	38	26	133	203	185	210
Spleen	227	185	121	212	122	125	87	138
Liver	3500	3741	3862	3234	3306	3325	2731	3075
Kidney	250	259	241	200	167	175	183	188
Adrenal	5	7	6	6	4	6	4	6

In the case of bilateral organs only one side was weighed except adrenal and kidney which are mean values. N.F. = not found.

Table 3. Effect of prednisolone on mean absolute white cell counts ($\times 10^9/l \pm$S.D.) *of pairs of pigs, A: before treatment; B: at end of treatment; Diff: rise or fall during treatment; % percentage change*

Prednisolone dose		Lymphocytes	Neutrophils	Eosinophils
Control	A	12.0±0.8	16.2±9.8	0.3±0.1
	B	10.3±0.1	11.5±6.2	0.2±0.3
	Diff	−0.7±0.8	−4.7±3.7	−0.1±0.4
	%	−5.8	−29.0	−33.3
2 mg	A	12.2±0.1	20.7±2.0	0.2±0.3
	B	9.3±0.1	8.7±2.0	0.4±0.3
	Diff	−2.9±0.1	−12.0±4.0	+0.2±0.1
	%	−23.8	−58.0	+100.0
4 mg	A	14.6±2.9	9.0±3.2	0.2±0.2
	B	10.0±1.3	5.7±1.2	0.5±0.6
	Diff	−4.6±1.6	−3.3±4.4	+0.3±0.8
	%	−31.5	−36.7	+150.0
6 mg	A	11.5±1.8	15.6±9.9	0.5±0.4
	B	6.6±0.6	8.5±4.4	0.8±0.7
	Diff	−4.9±2.5	−7.1±5.6	0.3±0.4
	%	−42.6	−45.5	+60.0

the control group because one pig had a pretreatment value of only 10.7 g/l compared with a mean of 24.1±S.D. 2.2 g/l for the other 7 pigs. If this latter figure is taken as a more acceptable pre-treatment value for the control group, then it is found that, whereas the control and 2 mg dosed groups showed mean increases in albumin of only 1.7 g/l (+7.05%) and 1.2 g/l (+4.8%) respectively, the 4 mg and 6 mg dosed groups showed mean increases of 9.0 g/l (+36.9%) and 7.8 g/l (+36.1%) respectively.

Table 4 also indicates that whereas the control group showed increases in mean total

Table 4. Effect of prednisolone on electrophoresis-derived mean serum protein values (g/l ± *S.D.*) *of pairs of pigs, A: before treatment; B: at end of treatment; Diff: rise or fall during treatment;* %: *percentage change*

Prednisolone dose		Serum albumin	Serum globulins			
			α	β	γ	Total
Control	A	18.6±11.1*	12.0±4.5	6.5±0.4	5.6±1.7	24.0±2.3
	B	25.5±3.7	17.5±2.8	12.3±2.6	9.2±5.4	39.0±0.1
	Diff	+6.9±7.4*	+5.5±7.2	+5.8±2.1	+3.6±7.1	+15.0±2.1
	%	+37.1*	+45.8	+89.2	+64.3	+62.5
2 mg	A	25.1±1.8	15.9±3.0	12.6±2.9	8.8±2.3	37.3±8.1
	B	26.3±4.4	16.8±2.1	12.2±1.6	10.4±1.8	39.4±2.0
	Diff	+1.2±6.2	+0.9±5.0	−0.4±4.5	+1.6±0.5	+2.1±10.1
	%	+4.8	+5.7	−3.2	+18.2	+5.6
4 mg	A	24.4±2.0	16.6±0.7	10.5±1.1	8.4±1.0	35.5±0.8
	B	33.4±0.9	13.5±1.1	10.1±3.7	8.5±0.4	32.1±3.0
	Diff	+9.0±1.1	−3.1±1.8	−0.4±2.6	+0.1±1.3	−3.4±2.2
	%	+36.9	−18.7	−3.8	+1.2	−9.6
6 mg	A	21.6±1.0	13.8±0.7	8.4±1.1	6.4±0.3	28.5±2.1
	B	29.4±2.3	13.8±0.2	9.3±0.9	7.6±1.6	30.6±0.6
	Diff	+7.8±1.3	0.0±0.5	+0.9±0.3	+1.2±1.9	+2.1±2.7
	%	+36.1	Nil	+10.7	+18.8	+7.4

*See Text.

globulin values and in all globulin fractions during the treatment period, the treated groups showed much smaller increases or even decreases in the corresponding values.

Since alpha-1 and alpha-2 globulins were not always distinguishable on the Phoroscope trace, it is not possible to give separate figures for these sub-fractions.

Total serum globulin values obtained by differences from chemical analysis of total proteins and albumin (but unconfirmed by the electrophoretic method because of inadequate samples) suggest that in the 4 mg and 6 mg treated groups the total globulins reached a nadir of about 20 g/l during the 4th week of treatment. A similar effect was noted in the 2 mg treated group but the nadir was not so low (*ca.* 24 g/l) and did not appear until a week later.

None of the serum inorganic elements showed any differences between groups which could be attributed to the treatments. In particular, there was no evidence of sodium retention nor of potassium or calcium depletion in the treated groups.

Cell-mediated response

It appears from Table 1 that during the last week of prednisolone treatment (42–43 days after sensitization) there is an anergy in the prednisolone-treated pigs as compared with the controls. There is a tendency for this anergy to be reversed in the 3 treated pigs after withdrawal of the drug, i.e. 81–82 days after sensitization.

Post mortem and histological findings

The most spectacular finding was in the thymus. At the end of the treatment period there was a dose-related reduction in relative weight of the thymuses of the treated pigs (Table 2, $r=-0.99$, df=2, $P=0.01$; $y=90-11.3x$). When compared with the control, the reduction amounted to 24, 58 and 72% in the 2, 4 and 6 mg dosed pigs respectively. As the thymus of this control was more involuted than would be expected in a normal pig of this age, these percentage reductions are probably underestimated. Histologically, the thymus lobule size of the treated pigs was reduced, the cortex being more involuted than the medulla, but the density of the cell population of both cortex and medulla was normal. The lymphoid involution was paralleled by a relative widening of the fat and connective tissue interlobular septa (Fig. 1).

In those pigs killed 5 weeks after treatment was discontinued, the relative thymus weights represented 156, 142 and 165% respectively of the corresponding control value. Despite this "overshoot", all were histologically normal (Fig. 2).

A less spectacular reduction in relative weights was found in the lymph nodes of

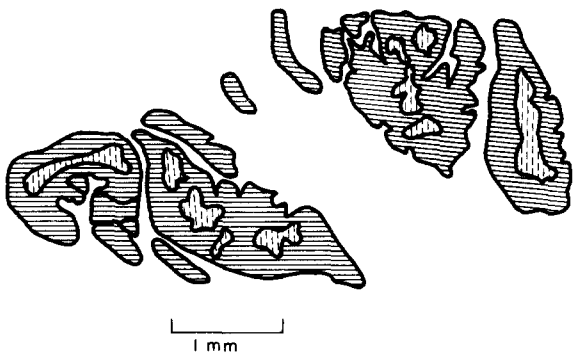

Fig. 1. Drawing of transverse section of whole thymus gland of pig receiving 6 mg/kg *prednisolone and killed at end of* 5 *weeks' treatment.*

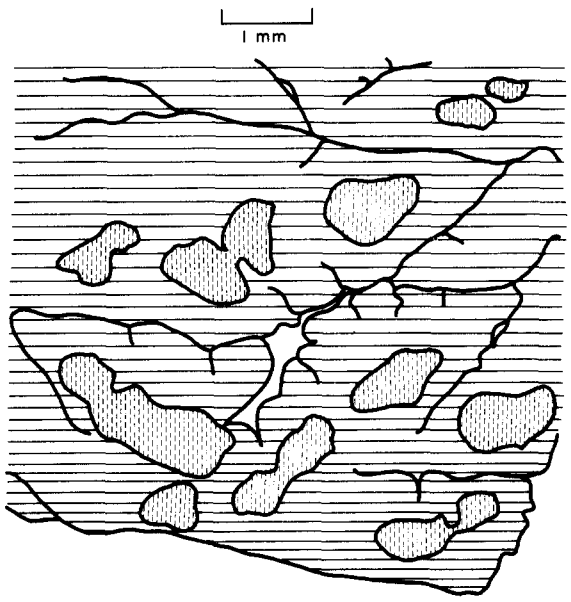

Fig. 2. Drawing of approximately half of cross section of thymus of control pig killed at end of experiment.

the treated pigs slaughtered at the end of treatment (Table 2). This was not dose-related but averaged 23% in the splanchnic nodes and 13% in the superficial nodes.

Histologically, the lymph nodes of the 6 mg dosed pig killed at the end of treatment, were distinctive in several respects. In contrast to the control and other treated pigs killed at the same time, (1) the submandibular node showed many "blast" cells with numerous mitotic figures both in the reaction centres and in some of the paracortical regions where there was a reduction in small lymphocytes (Figs. 3 and 4); (2) the prescapular node had virtually no lymphocytes in the cortex (Figs. 5 and 6); (3) the medulla of the mesenteric lymph node was reduced in size and the paracortical zone was more extensive than usual (Figs. 7 and 8); (4) the external iliac node showed more foci of megakaryocytes and normoblasts in the medulla; (5) the Peyer's patches were the smallest in the series and the cells in the open centre were inactive (Figs. 9 and 10). This animal had similar amounts of adipose tissue to the others, its poor weight gain being attributable to poor bone and muscle development.

There was no evidence of "overshoot" in the relative lymph node weights of the pigs killed 6 weeks later; indeed, although the superficial nodes were now comparable in relative weight to the control nodes, the splanchnic nodes averaged only 72% of the relative weight of the control nodes. Histologically, the external iliac lymph nodes of all pigs killed at this time showed more extramedullary haemopoiesis than in any of those killed earlier. In addition, the ratio of cortex to medulla was reversed from 1:3 to 3:1, this being attributable to an increase in size of both the reaction centres and the paracortical zone.

There was some variation in the relative weights and in the histological appearances of the white pulp of all 8 spleens, but these were not correlated with dose of prednisolone nor timing of death; nor were the variations in the white pulp related to the weight of the organ.

Lung, liver, kidney, adrenal and stomach were normal and similar in all cases. No pig showed any evidence of ascites or osteoporosis.

The bone marrow samples showed only differences that were attributable to age and growth rate. The core biopsies were comparable to the post mortem specimens.

The only evidence of intercurrent disease was in the second control pig which had a mild inflammation of the caecum associated with spirochaetes.

DISCUSSION

The extent and rate of development of the lytic effects of prednisolone on the lymphoreticular system of the pigs, in particular the thymus, the lymph nodes and the blood lymphocytes, clearly place them in the corticosteroid-resistant group of species which includes man, ferret and guinea pig, as opposed to the corticosteroid-sensitive mouse, rat, hamster and rabbit, in which the lytic effects are much more severe and rapid at comparable dosage [3].

The reduction in size of the thymus during treatment and its recovery with "overshoot" after withdrawal of treatment is a phenomenon



Fig. 3. Submandibular lymph node of pig killed at end of treatment with 6 mg/kg *prednisolone. Note large "blast" cells in the nodule and in the paracortical zone where there are fewer small lymphocytes than in Fig. 4. H & E* ×250.

Fig. 4. Submandibular lymph node of control pig. Compare with Fig. 3. H & E ×250.

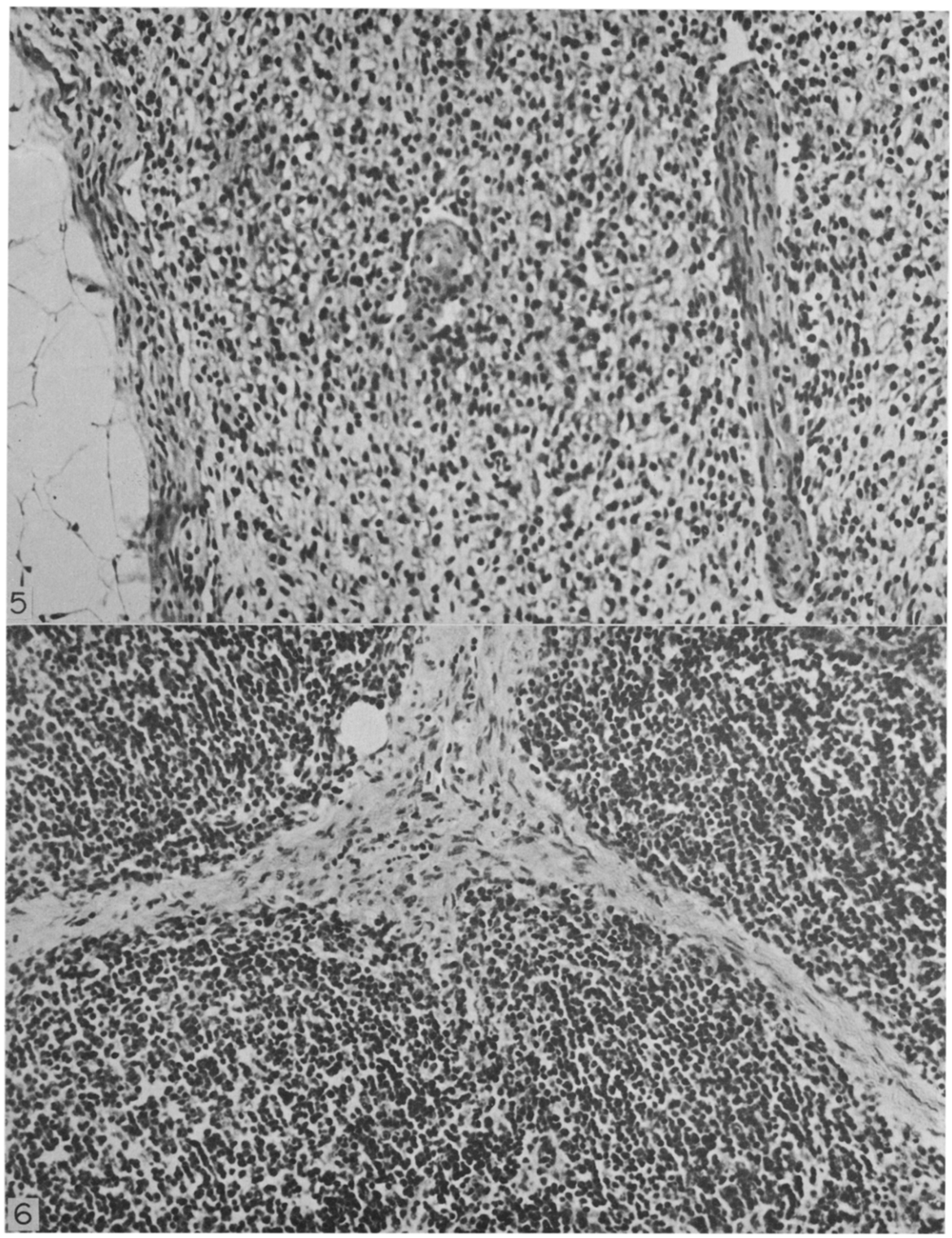

Fig. 5. Prescapular lymph node of same pig as Fig. 3. Note no lymphocytes round trabecula. There is no difference in the fixed histiocyte meshwork of cortex and medulla. H & E ×250.

Fig. 6. Prescapular lymph node of pig killed at the end of treatment with 2 mg/kg *prednisolone. Note modules and paracortical zone round trabeculae. Compare with Fig. 5. H & E* ×250.

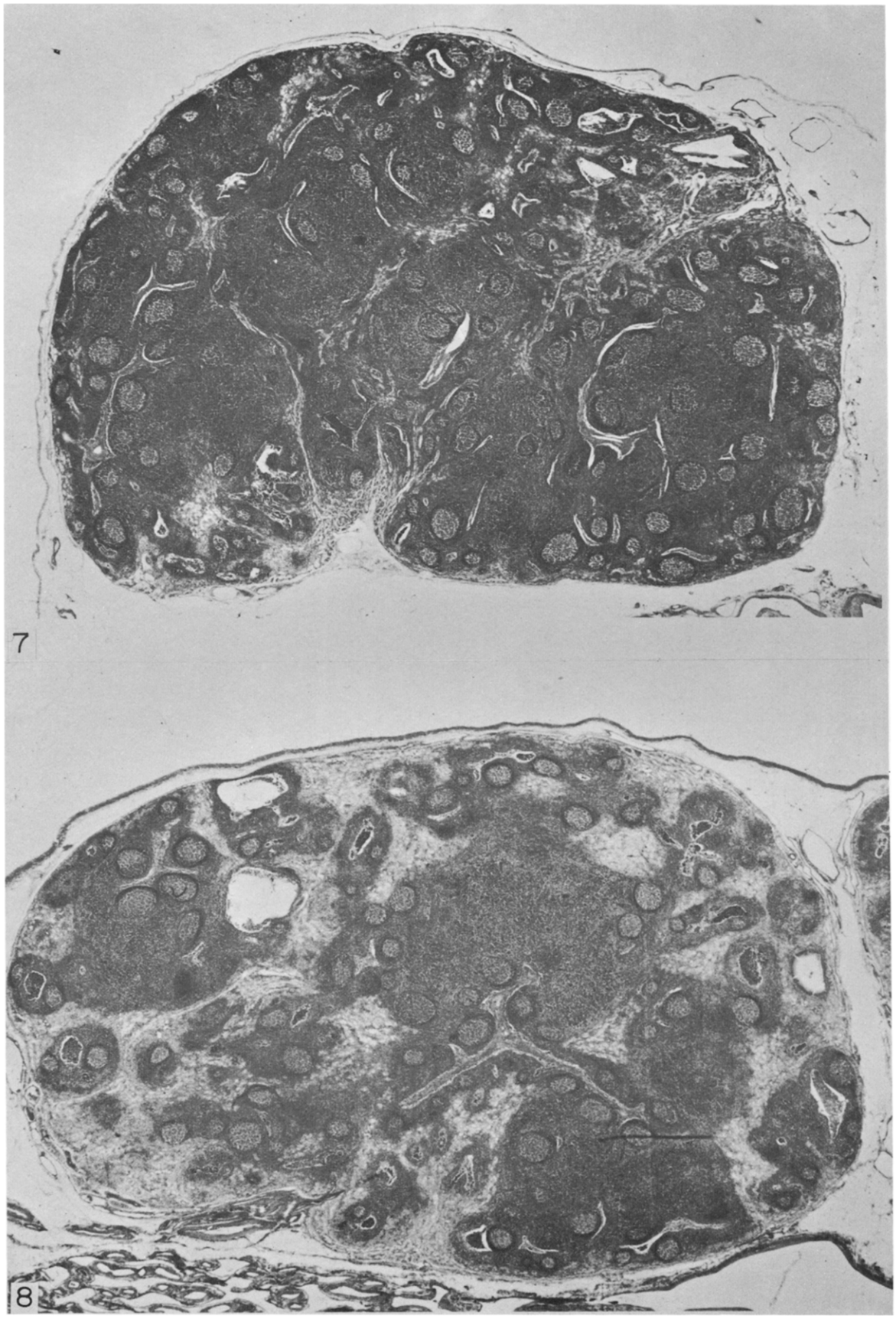

Fig. 7. Mesenteric lymph node of same pig as Figs. 3 and 5. Note small size of medulla and increased size of paracortical zone. H & E ×14.

Fig. 8. Mesenteric lymph node of control pig. Compare with Fig. 7. H & E ×14.

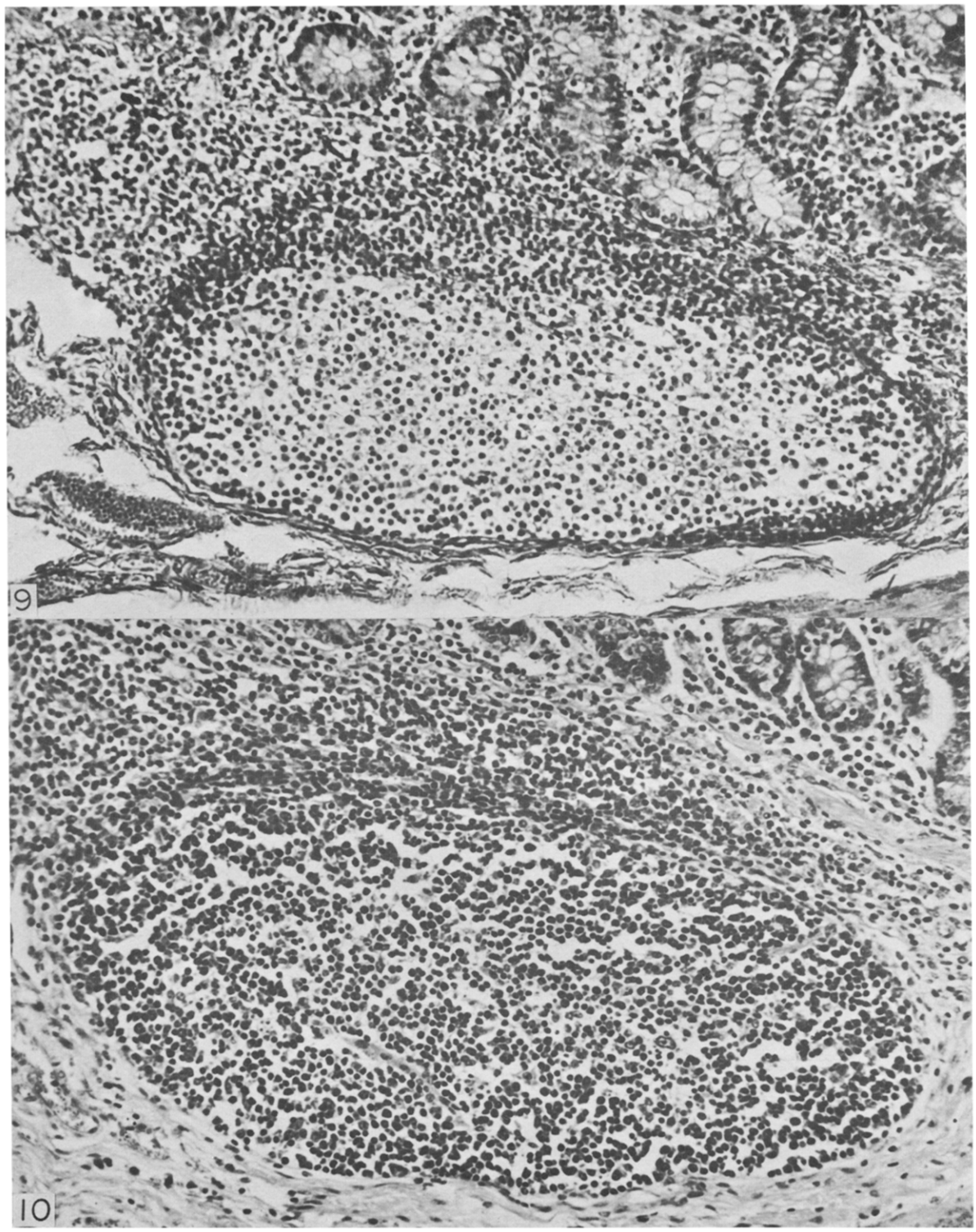

Fig. 9. *Peyer's patch on the ileum of same pig as Figs.* 3, 5 *and* 7. *Note depleted "inactive" nodule. H & E* ×250.

Fig. 10. *Peyer's patch from the ileum of control pig. Compare with Fig.* 9. *H & E* ×250.

which has been described in non-lymphomatous children treated with prednisone or triamcinolone [10]. The growth retardation observed in the 6 mg dosed pigs has also been encountered in corticosteroid-treated children [11]. The latter usually show a rapid increase in growth after treatment is discontinued but this effect was not observed in any treated pigs during the 5 weeks post-treatment period. The growth retardation observed suggested that a dose of 4 mg/kg/day might be more suitable than 6 mg/kg/day for the therapeutic study which followed.

In the absence of any evidence of ascites, the pot belly observed at the two higher levels was presumed to be attributable to muscle relaxation. This was supported by the fact that the apparent distension disappeared when an affected animal was laid on its back.

The observed depression of serum globulin and increase in serum albumin levels associated with treatment have also been observed in man but mostly in relation to corticosteroid therapy of chronic diseases. It is popularly believed that corticosteroids affect especially the γ-globulin fraction but Bernfeld *et al.* [12] demonstrated that in ACTH-treated patients the beta fraction was mainly affected.

In our own experiments, the effects of prednisolone treatment on serum proteins were more obvious in lymphoma cases than in normal pigs. For this reason these effects will be discussed more fully in a later paper.

With respect to corticosteroid suppression of cell-mediated immunity, the pigs again fell within the steroid-resistant group of species since it has been shown that, although corticosteroids can suppress skin sensitivity reactions in that group, large doses or prolonged treatment are required. In man, for example, prednisone given orally in 40 mg/day doses had to be administered on average for 13.6 days to negate the effects of the PPD tuberculin test [13].

The immediate neutrophilia and eosinopenia which are to be expected in man receiving over 10 mg prednisone per day and which persist while treatment lasts [11] were not observed even transiently in any of the treated pigs. We have, however, observed neutrophilia in pigs treated with corticosteroids in another connection.

It is thus evident that the reactions of normal pigs to prednisolone show promising similarities to those occurring in man. Further, no laboratory animal in common use is also comparable to man both in size and in being omnivorous. Young pigs are relatively easily handled and yet are large enough to permit sizeable samples of blood and biopsy materials being taken repeatedly without ill effect. As will be emphasised in a later paper, the survival time of lymphomatous pigs is much closer to that of children than is that of small rodents. All these features point to the practicability and advantages of using the pig as a model for the testing of cytotoxic drugs.

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Reversible Morphological Changes in Human Breast Fibroblasts Exposed to Stilboestrol*

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Abstract—*Fibroblast cultures derived from human breast material treated with pharmacological doses of stilboestrol* (115 μm) *undergo a morphological transformation to an epithelial appearance. The effect is rapid and reversible. It is independent of RNA and protein synthesis and is not inhibited by an oestrogen antagonist* (*tamoxifen*).

INTRODUCTION

IN THE course of long-term studies on the *in vitro* behaviour of human breast tumour cultures we have examined the effects of steroids and steroid analogues on fibroblast strains derived from explants of tissue into fibrin clots to try to establish whether the response of these connective tissue cells is distinct from that of the predominantly epithelial primary cultures previously reported by us [1]. We report here observations on fibroblast strains from breast cancer, fibroadenoma and chronic cystic disease of the breast treated *in vitro* with the synthetic oestrogen analogue, stilboestrol, which may shed some light on the report published by Bishun and colleagues [2] drawing attention to a change in morphology of cells in fibroblast-dominated cultures derived from breast tumours.

MATERIAL AND METHODS

(*a*) *Culture medium*

In all the experiments referred to the cells were grown in a medium composed of Eagle's minimum essential medium (MEM) buffered with bicarbonate and Hepes, containing penicillin and streptomycin 100 units per ml., L-Glutamine 2mM and supplemented with foetal bovine serum (1, 10 or 20% as stated), all obtained from Flow Laboratories, Irvine, Scotland.

(*b*) *Chemicals*

A sterile aqueous solution of α,α′-diethyl-4,4′-stilbenol disphosphoric acid ester was obtained from Ward Blenkinsop and Co. Ltd. under the name of Honvan (250 mg per 5 ml ampoule). This was used in a dose of 10 μl per 10 ml of medium giving a final concentration of 115 μm and administered once with each medium change except when stated otherwise. Tamoxifen was kindly supplied by Dr. A. H. Todd of I.C.I. Pharmaceuticals Division. Sterile solutions in ethyl alcohol were used in a dose of 10 μl per 10 ml of medium giving final concentrations of 10^{-4} 10^{-5} and 10^{-6} M. Oestradiol and testosterone were used as described in [1]. Actinomycin D (Merck, Sharp and Dohme International) in aqueous solution containing 10 mg/ml mannitol, and cycloheximide (Sigma) in phosphate-buffered saline, were added to the culture medium to give final concentrations of 5 μg/ml and 50 μg/ml respectively. Colchicine (B.D.H.) dissolved in distilled water and filter sterilised was added to give a final concentration of 0.12 mg/ml of medium.

(*c*) *Cells*

The cells used in this investigation were derived from explants of human breast tissue removed at operation. The material was placed in serum free MEM containing penicillin and streptomycin and kept at 4°C. Within 24 hr of removal the tissue was chopped into small fragments and mechanically disrupted

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for 3 sec in a Colworth "Stomacher 80" and released cells filtered through fine gauze. The residual tissue was cut into pieces measuring about 1 mm^3, placed into polystyrene tissue culture flasks (Corning) and secured by a fibrin clot formed by mixing 3 vol of citrated human plasma with one volume of a solution of calcium chloride (2% aqueous) and placing a drop on the explanted fragment. In early experiments the entire surface of the flask was coated with fibrin but this was found to be unnecessary and wasteful. When the clot had formed 10 ml culture medium supplemented with 20% serum was added. Cells migrating into and growing in the fibrin meshwork had the typical bipolar morphology of fibroblasts and most explanted fragments gave rise to these fibroblastic outgrowths, in particular those portions which included adipose tissue. Fragments of tissue from carcinomas, fibroadenomas, chronic cystic disease of the breast and two cases of gynaecomastia were cultured. All these gave rise to fibroblasts although the details of their behaviour differed in some respects, as will be described elsewhere (Riley, Latter and Sutton, manuscript in preparation). Flasks showing dense fibroblastic outgrowths were trypsinised (trypsin 10% in a buffered Ringers solution) and the cells resuspended and inoculated into further flasks to which fresh medium was added. Stocks of cells were built up by subsequent passages and representative cells frozen in liquid nitrogen at about the fifth passage. The experiments reported here were made on cells between the passage numbers 3–16 and the tabulated values, given in the Results, obtained from the 3rd–7th passage after liberation from the fibrin clot. For comparison of the effects of stilboestrol some other cultures were also tested, including human fibroblastic cells from a biopsy of an axillary lymph node, the human foetal lung fibroblast line MRC5 (obtained from Dr. J. P. Jacobs) and the murine fibroblastic line 3T3 (obtained from Dr. R. Weiss). These cells were cultured in Hepes-buffered MEM with 10% foetal bovine serum supplementation. Subculturing in all cases was by trypsinisation, the cells being washed and resuspended in 1 ml of medium. Cell counts were made on 20 μl aliquots suspended in 10 ml 0.9% saline using a Coulter counter (Model A) calibrated to register particles with a spherical diameter greater than 3 μm. On the basis of the counts the stock cells were diluted with medium to give calculated seeding densities and inoculated into culture flasks. Doubling times were calculated for cells cultured in the presence of 1, 10 and 20% serum and when stilboestrol was added to the culture medium. Morphological changes were recorded by photographing representative areas using a Nikon inverted photomicroscope with phase-contrast optics. Changes in one culture (FDF/Ed) were filmed using a Vinten time-lapse Camera (MK. II) mounted on an inverted microscope. The result of adding stilboestrol and the effect of subsequent washing and change to normal medium was recorded on Ilford FP4 film using intermittent green-light illumination with a lapse interval of one minute per frame.

RESULTS

(1) *Morphological alteration*

In all instances a profound and persistent change in the morphology of the cells was observed within 24 hr after the addition of stilboestrol to the culture medium (Fig. 1). The degree of alteration varied between cultures, being most marked in the fibroblasts derived from chronic cystic disease (CCD) and carcinomas of the breast. To some extent this might be related to the initial appearance of the cells which we have found to be more characteristically fibroblast-like (i.e. elongated, bipolar cells with small oval nuclei) in fibroblastic cultures from chronic cystic disease and cancers than those derived from fibroadenomas of the breast. The latter resemble 3T3 cells more than MRC5's in being more spread out on the substratum. The major morphological features of the stilboestrol-treated cells are that they are more cuboid and resemble epithelial cells: such an alteration is more visually dramatic from an initial bipolar appearance than if the cells are already relatively spread out, and in this sense it might be said that the fibroadenoma fibroblasts have the normal appearance of being partially affected by stilboestrol and this is also true for their growth behaviour (see below). Although the strain of fibroblasts from the axillary tissue was less responsive to stilboestrol than those derived from breast tissue the fact that both MRC5 and 3T3 cells were sensitive to the agent (Table 1) excludes the possibility that in general mammary fibroblasts are differentially sensitive.

Reversibility of morphological change

The reversibility of the effect of stilboestrol addition was shown for cells which were subcultured by trypsinisation and reinoculated into normal culture medium (Fig. 1c). That

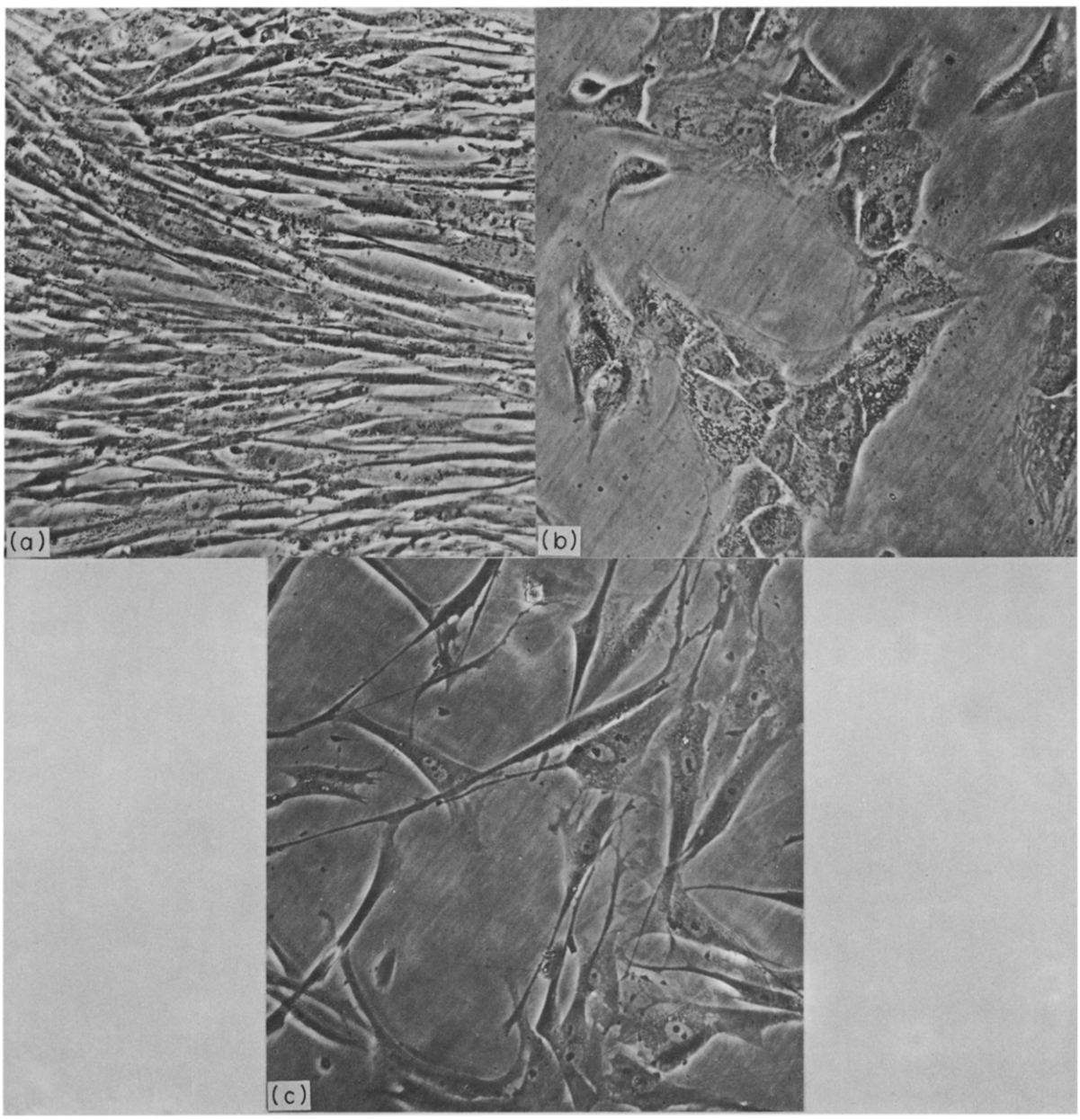

Fig. 1. *Fibroblast culture before* (*a*) *and five days after* (*b*) *stilboestrol addition to the medium. In addition to this obvious change to an epithelial morphology there is increased cytoplasmic vacuolation.* (*c*) *Reversion of cells to fibroblastic shape after trypsinisation and re-inoculation into control medium* 18 hr *after reinoculation.*

Table 1. *Mean shape indices of control and stilboestrol-treated cultures*

Cell strain	Mean shape index (Treatment time in days)		
	Control	+stilboestrol	Post stilboestrol
NDF (9)	11.8	2.7 (1)	4.3 (2)
		1.3 (5)	
		1.4 (8)	
CDF (8)	12.1	2.2 (1)	
		2.0 (8)	3.4 (1)
FDF (6)	8.6	1.9 (1)	
		2.0 (6)	5.5 (1)

The shape index was estimated as described in the text and illustrated in Fig. 2. The numbers in parentheses indicate the numbers of cultures on which observations were made.
NDF = normal breast tissue-derived fibroblast cultures;
CDF = cancer-derived fibroblasts;
FDF = fibroadenoma-derived fibroblasts.

this reversibility was not contingent upon the action of trypsin was shown for cultures which were washed twice with medium after removal of the stilboestrol-containing medium and before the introduction of control medium. This proceedure was also employed in the case of the culture observed by time-lapse cinemicroscopy which showed a rapid return to the usual bipolar fibroblastic morphology.

Rapidity of effect

Changes were observed within 18 hr of addition of stilboestrol and were very marked in all the treated cultures by 48 hr. Examination of the time-lapse film indicated that some reduction in the localisation of the leading lamella occurs in some cells within a few minutes of the addition of stilboestrol. The cells rather gradually assume their final epithelial appearance, although some cells, which seem to be more sensitive, round up and show circumferential blebbing. In growing cultures the cells continued to undergo mitosis normally except that the division products reattached and spread on the culture surface less rapidly than control cultures. To what extent this is the result of competition for space on the culture surface is not clear. Experiments with actinomycin D or cycloheximide present in the medium in concentrations sufficient to inhibit RNA or protein synthesis by more than 95% during stilboestrol treatment showed that the effect of stilboestrol is independent of new synthesis.

Reversibility

The alteration in morphology was found to revert rapidly on withdrawal of stilboestrol. The time-lapse film showed almost total reversal in 30–60 min after washing out the agent.

To try to quantify the major morphological change (i.e. that of the shape of the general outline of the cell) measurements of cell diameters were made and expressed as a ratio of the longest diameter to the orthogonal diameter at its midpoint. This crude shape parameter, illustrated in Fig. 2, gives a measure of the shape change. Mean values for representative cultures are given in Table 1. Some indication of the rates of shape change was obtained by plotting against time the ratio of these measurements made from the projected image of single cells at various intervals (see Fig. 3). Reversal of the morphology was not dependent on RNA or protein synthesis, as shown by the failure of actinomycin D and cycloheximide to inhibit the reassumption of bipolar shape when stillboestrol was removed.

Effect on the doubling time

The overall growth rates of the cultures, assessed by the calculated doubling times were found to be extremely variable for different

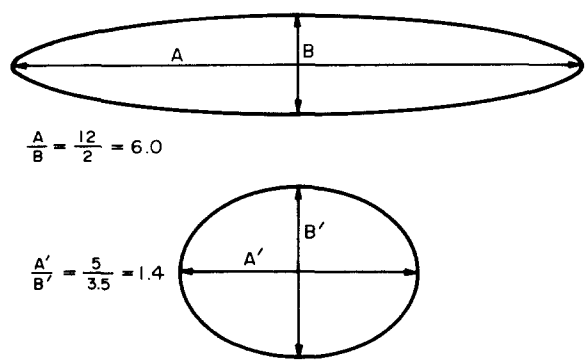

Fig. 2. *Estimation of simple morphological parameter. The ratio of the longest cell diameter* (*A*) *to the diameter orthogonal to it at the midpoint* (*B*) *gives values of* 6.0 *and* 1.4 *for the upper and lower shapes respectively.*

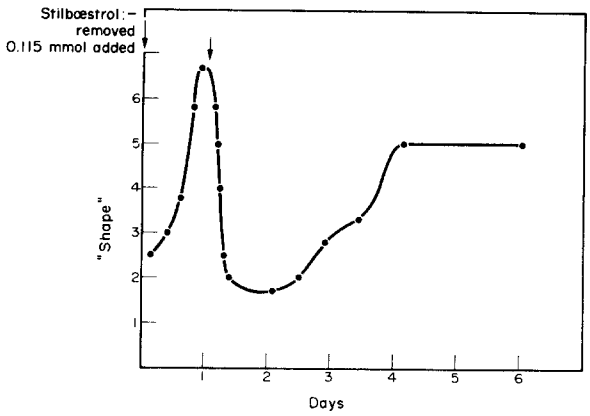

Fig. 3. Shape change of cells exposed to and removed from effects of stilboestrol. Data from time lapse films.

cultures. In general the doubling times for fibroblasts derived from non-tumour material (NDF cultures) were shorter than cancer-derived fibroblast (CDF) cultures. The fibro-adenoma-derived fibroblasts (FDF) included in these experiments grew very slowly. The growth indices (reciprocal doubling times) are tabulated in Table 2 and show that stilboestrol addition to the medium is inhibitory to the growth of the cells. The mitotic rate was lower in treated cultures but part of this effect must be ascribed to loss of cells by displacement from the culture surface giving rise to negative values. Recovery after stilboestrol treatment in the case of cells trypsinised and re-inoculated into fresh medium was found to be less rapid than indicated by the rate of shape recovery in the filmed culture but this may have been due to the fact that the seeding density in these cultures was often of necessity lower than the optimum with resulting lag in the initiation of growth. Reattachment of the cells appeared to occur normally and this was also found in two instances where treated cells were trypsinised and reinoculated in medium containing stilboestrol.

DISCUSSION

Our results show that stilboestrol present in culture medium in a dose of 115 μm produces morphological changes in fibroblasts from several sources. The changes are reversible. The alteration in shape makes the cells resemble epithelial sheets. Since the effect was found to be independent of RNA and protein synthesis we conclude that it is not exerted through an inter-action with specific oestrogen receptors. This view is consistent with the responsiveness of all the tested cells, irrespective of their origin, and also with the failure of the oestrogen antagonist Tamoxifen to inhibit the response. Although it is possible that more than one class of oestrogenic receptor exists, particularly in view of recent reports of uptake of radio-actively labelled oestrogens by the nuclei of myocardial cells [3], specific oestrogen receptors are saturated in the nano- to pico-molar range of oestrogen concentrations [4] whereas the effects which we have observed are detected at much higher doses. Similar findings with oestrogens have been reported by Lippman *et al.* [5] in relation to thymidine incorporation by MCF-7 human breast cancer cells *in vitro*. They found that at concentrations of oestradiol greater than 0.1 μm, thymidine incorporation is inhibited even by the 17-α analogue which does not bind to the oestrogenic receptors. The change in cell shape is associated with a reduction in growth rate and an increase in cytoplasmic vesiculation. It is probable

Table 2. Effect of stilboestrol addition on the generation times of fibroblast strains

Cell strain	Mean growth index		
	Control	+stilboestrol	Post stilboestrol
NDF	0.15	−0.12 (3)	0.01
CDF	0.07	−0.11 (3)	0.04
FDF	0.02	−0.16 (1)	−0.01
2°CDF	0.15	0.03 (1)	—
MBDF	0.26	−0.18 (1)	0.27
3T3	1.81	0.28 (1)	0.81
MRC5	0.59	0.20 (1)	—

The growth indices represent the reciprocal of the mean doubling time of the cultures in days. In addition to the cells tabulated in Table 1. there are: 2°CDF = fibroblasts from secondary carcinoma in an axillary lymph node; MBDF = male breast-derived fibroblasts; 3T3 is the Swiss mouse line of fibroblastic cells and MRC5 is a diploid human fibroblastic line.

that these stilboestrol effects are unrelated to oestrogen receptor binding but due to inter-action of the compound with one or several other components of the cell. The hydrophobic properties of steroid hormones and their analogues make it likely that the cell membrane will adsorb significant amounts. Uptake measurements at heptane/water interfaces give a distribution coefficient of 2.3 for diethylstilboestrol indicating a high preference of the compound for a hydrophobic environment and saturation of the interface occurs when the concentration in the aqueous phase reaches about 5×10^4 molar after which there is a linear decline in the interfacial tension [6]. It is possible that in the concentration range we have used it is this detergent effect of stilboestrol which is demonstrated, acting either on the plasma membrane or some protein component of the cells which determines their morphology in monolayer culture. Evidence favouring some membrane effect was obtained by the observation that the addition of hydrocortisone after stilboestrol treatment induced a marked vesiculation of the cells.

Alternatively, the non-specific action of stilboestrol could be exerted through an effect on cytoskeletal elements, particularly in view of its action in inhibiting mitosis. Lettré [7] was the first author to describe the inhibition of mitosis produced by oestrogenic agents on cultures of chick heart fibroblasts exposed to diethylstilboestrol and disodium oestradiol phosphate in concentrations exceeding 40 μg/ml in the culture medium. Since this effect required ten times the dose of α-phenyl-β-(p-methoxy phenyl) ethylamine Lettré suggested that an aminated derivative of oestrogens might be more effective and attempted to show a general structural relationship between adrenalin, colchicine and sterols. The morphological alterations induced by exposing the fibroblasts used in our experiments to colchicine were qualitatively similar to those induced by stilboestrol. We examined transmission electron micrographs of our material which failed to show the usual microtubular structures, but while this is consistent with the view that the reported action of stilboestrol is by a non-specific interference in microtubule assembly it does not constitute proof of the mechanism involved. Since stilboestrol takes several hours to manifest its full effect (i.e. it has a much slower action than colchicine) and the time-lapse film shows cells completing mitosis for up to 12 hr after the beginning of exposure we suggest that stilboestrol may undergo metabolic conversion to a form which affects cell division.

It is highly probable that the effect on the cell membrane or the cytoskeleton which we suggest is an action ascribable to natural steroid hormones as well as synthetic compounds such as Tamoxifen if they are used in pharmacological concentrations. For example, Bishun *et al.* [2] have described what appear to be similar morphological changes in human breast carcinoma-derived fibroblasts grown in a medium containing 18.3 μm 17-β oestradiol.

In our system androgens did not differ from oestrogens in their effect whereas Hsie and Puck [8] have shown a fibroblastic transformation in Chinese hamster ovary cells in the presence of androgens and relate this to effects on cyclic nucleotide concentrations. It is probable that different cell types respond in differing ways to exposure to high concentrations of steroid hormones.

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Non-Hodgkin Malignant Lymphomas. Clinico-Pathologic Correlations with the Kiel Classification

Retrospective Analysis of a Series of 274 Cases

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Abstract—*Three hundred and sixty case histories of patients followed for non-Hodgkin lymphoma between 1965 and 1976 were reviewed from the histologic point of view for classification according to Kiel's nomenclature. The diagnosis was rectified for 86 patients and confirmed for the 274 remaining ones; these latter cases were classified and their clinico-pathologic and evolutive correlations studied. The most striking fact is a very distinct difference between the so-called low grade forms, which have a non-corrected median survival of four and a half years, whereas in the high grade forms it is 7 months. Except for a few minor differences, the observations noted in this series cross-check those previously published.*

INTRODUCTION

The HISTOLOGIC classification of non-Hodgkin malignant lymphoma (NHML) has been, for a long time, submitted to numerous variations. During the last few years the most frequently used classification has been the one of Rappaport [1]. As for as we are concerned, we have applied this classification for several years without obtaining a distinct clinico-pathologic correlation [2]. We therefore decided to try Kiel's new classification, first proposed by Lennert [3], then by several European pathologists [4] in order to test its validity. This classification was applied, in a retrospective manner, to all cases observed during the last 12 yr; clinico-pathologic and evolutive correlations were particularly investigated. The results of this study are presented in this paper.

MATERIAL AND METHODS

This study included 434 case histories (with histologic documents that could be reviewed) of patients treated and followed up at the Fondation Bergonié for NHML between January 1965 and December 1976.

Seventy-four cases were eliminated due to insufficient material for review (incomplete specimens, tissue sections improperly fixed or too thick, only one slide). Consequently only 360 case histories could be considered.

Throughout the period studied the patients were submitted to the same simple routine investigations according to the recommendations of Rye's symposium for Hodgkin's disease [5] with a complete clinical examination, a thoracic X-ray and tomography of the mediastinum, an abdominal lymphography and the usual biologic investigations. There were no exploratory laparotomies. Bone marrow biopsy was done only when clinical and/or biological data were considered suspicious for bone marrow involvement.

On the other hand, the treatments varied considerably during the period studied, with particular improvement in chemotherapy and more frequent indication of radio-chemotherapeutic associations; but these treatments were never conditioned by the histologic type.

All histologic specimens were examined after routine staining by Hematoxylin-eosin, Gomori, PAS and Giemsa. In all cases the specimen reviewed was the one on which the initial diagnosis was based. The pathologist who reviewed the slides knew the age of each patient and the site of the biopsy.

For 86 patients the initial diagnosis of NHML was modified as indicated in Table 1. For the 274

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Table 1. Diagnosis of 86 excluded patients

73 malignant
40 carcinomas
27 undifferentiated
1 adenocarcinoma
1 Regaud lymphoepithelioma
11 undetermined
18 blood malignancies
6 malignant histiocytosis
5 lympho-epithelioid lymphomas
2 mycosis fungoides
2 angio-immunoblastic lymphadenopathy
3 miscellaneous
15 miscellaneous and undetermined malignancies
13 benign

Table 2. Kiel classification

Low grade
Lymphocytic (LC)
Lymphoplasmocytoid (LP)
Centrocytic (CC)
Centroblasto-Centrocytic (CB-CC)
High grade
Centroblastic (CB)
Lymphoblastic (LB)
Immunoblastic (IB)
Unclassifiable

Table 3. Distribution of histologic types

Low grade		165
Lymphocytic	23	
Lymphoplasmocytoid	31	
Centrocytic	10	
Centroblasto-centrocytic	101	
High grade		91
Centroblastic	2	
Lymphoblastic	39	
Immunoblastic	50	
Unclassifiable		18

cases in which the diagnosis of NHML was confirmed, Kiel's classification was applied (Table 2).

Fifty of these patients had been priorly treated and were taken in charge by the Fondation Bergonié at the time of a relapse; the other 224 were seen as soon as the diagnosis was established, prior to any treatment.

RESULTS

The distribution of the 274 NHML, confirmed by the histologic revision, according to Kiel's classification, is indicated in Table 3. The most important groups of centroblasto-centrocytic (CB-CC) lymphomas could be divided into three subgroups: nodular CB-CC (59 patients); nodular CB-CC with sclerosis (18 patients); diffuse CB-CC (24 patients).

The distribution according to age and sex is shown in Fig. 1. Low grade lymphomas are rarely observed before 30 yr and never before 20 yr; the sex ratio is 1.2.

The distribution by clinical stage prior to any treatment is indicated in Table 4. On one hand the patients are equally divided between stages I and II and stages III and IV. There is no difference according to the histologic type, except for lymphocytic forms where stages III and IV predominate and lymphoplasmocytoid forms where stages I and II predominate.

Table 4 gives the main initial tumoral sites. The frequency of visceral localizations and Waldeyer's ring in lymphoplasmocytoid forms and of mediastinal lesions in lymphoblastic forms, may be pointed out. On the whole, lymph nodes opacified by lymphography, are more often involved in low grade forms than in high grade ones.

During evolution, 19 secondary leukemias were observed; 9 in the LC, 3 in the CB-CC, 7 in the LB and 1 in the unclassifiable.

The distribution of 23 cases with positive Coombs' test and 4 cases with monoclonal dysglobulinemia shows no significant difference.

Table 4. Distribution by clinical stage

	Low grade				High grade				
Stages	LC	LP	CC	CB-CC	CB	LB	IB	Unclassifiable	Total
I	4	10	1	26	0	9	12	3	65
II	2	13	2	18	0	12	13	3	63
III	9	3	5	37	1	8	13	8	84
IV	6	4	1	15	1	10	8	2	47
Undetermined	2	1	1	5	0	0	4	2	15
Total	23	31	10	101	2	39	50	18	274

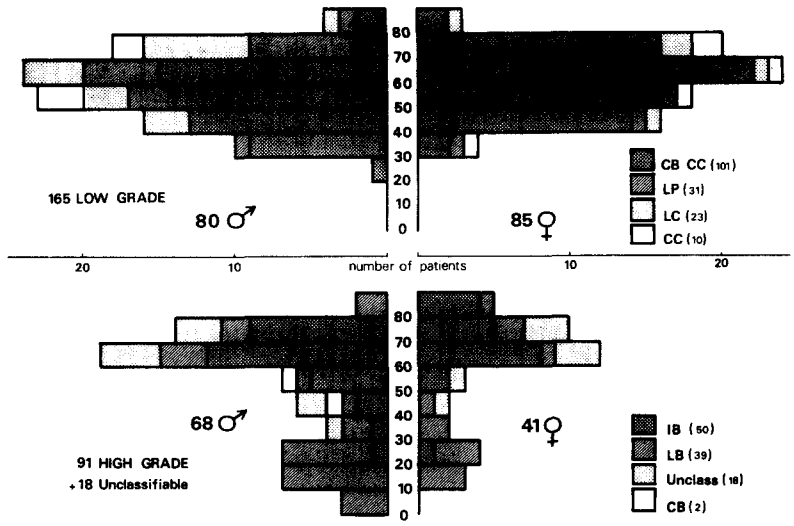

Fig. 1. Distribution of main histologic subtypes according to age and sex.

Table 5. Initial localization

	Low grade				High grade				
	LC	LP	CC	CB-CC	CB	LB	IB	Unclassifiable	Total
Total	23	31	10	101	2	39	50	18	274
Waldeyer	4	8	4	11	0	3	7	4	41
Mediastinum	4	1	1	12	1	17	4	5	45
Spleen	5	2	2	14	1	4	1	3	32
Lymphography	11	6	4	51	2	10	21	11	116
Visceral localization	3	18	0	8	1	13	15	1	59
Bone marrow	7	1	1	8	0	7	1	2	27

Table 6. Survival of patients as of April 1st 1977

	Low grade			High grade				
	LC	LP	CB-CC	CB	LB	IB	Unclassifiable	Total
Alive in complete remission	6	11	44	0	7	4	2	72
Alive with evolutive disease	6	5	10	1	2	3	1	28
Dead of lymphoma	4	12	40	1	27	38	8	130
Dead of intercurrent disease	3	2	4	0	0	2	3	14
Dead of undetermined cause	4	1	3	0	3	3	4	18
Total	23	31	101	2	39	50	18	274

The evolution is indicated by uncorrected actuarial survival curves. The state of the patients as of April 1st 1977 is indicated in Table 6. There is a high death rate due to intercurrent causes or to undetermined cause in relation to the old age of many patients. The Figs. 2–5 show a very clear difference between the low grade forms which have a median survival of 4.5 yr whereas that of the high grade ones is of 7 months, closely related to the unclassifiable forms. There is no definite difference between the main subgroups for the high grade (Fig. 3) nor for the low grade (Fig. 4). On the contrary, the subdivision of the CB-CC forms shows a less favorable evolution for the diffuse forms ($0.025 < P < 0.05$) situating them as intermediary between the high grade and the low grade, with a median survival of 2 yr.

Lastly, Fig. 6 shows the evolution of survivals for the 224 patients who received their first treatment at the Fondation Bergonié, in function

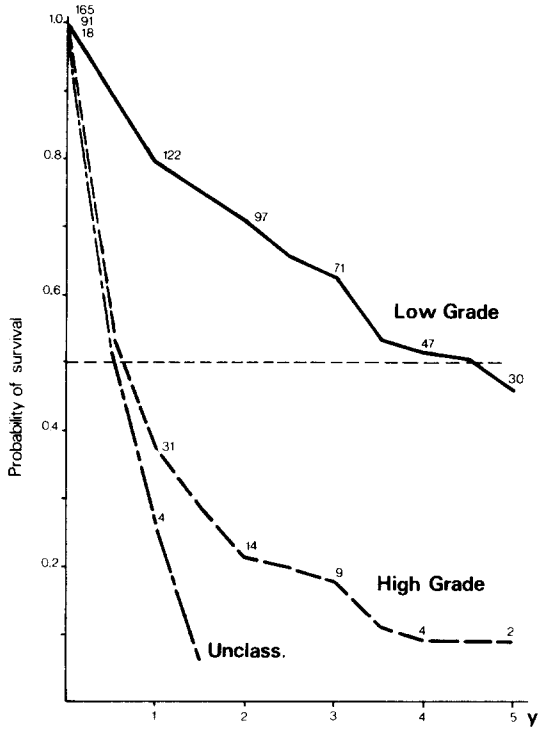

Fig. 2. Crude actuarial survival curves for all patients.

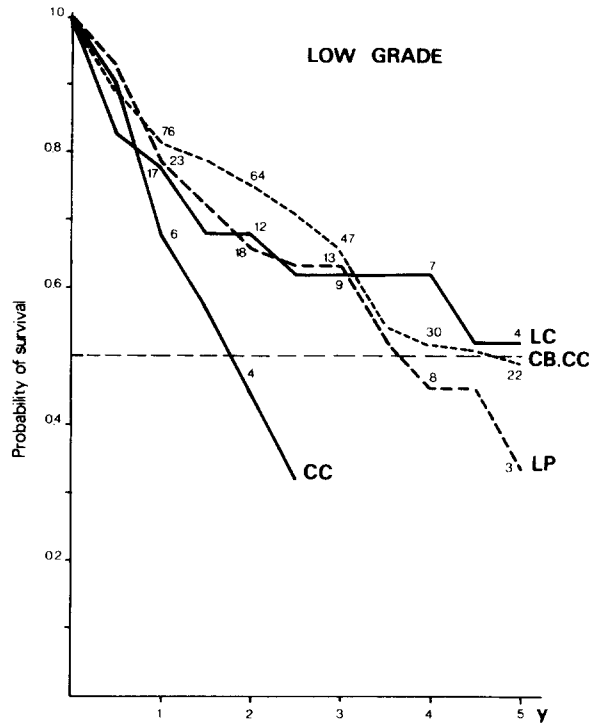

Fig. 4. Crude actuarial survival curves for low grade types.

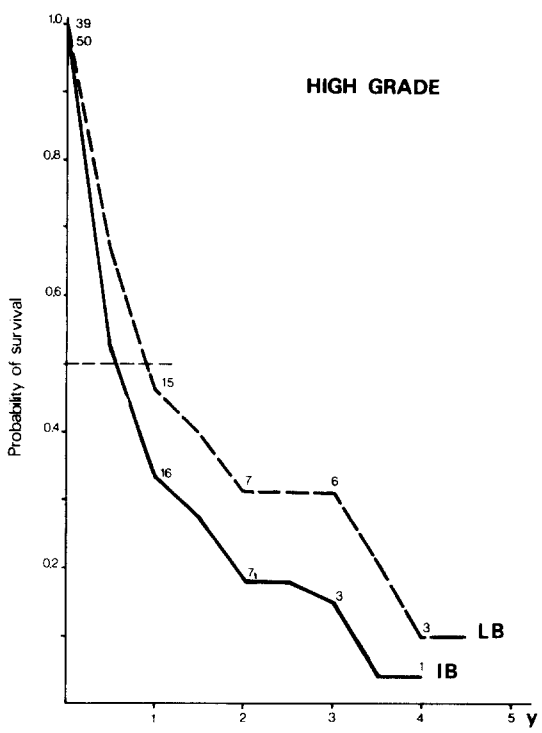

Fig. 3. Crude actuarial survival curves for high grade types.

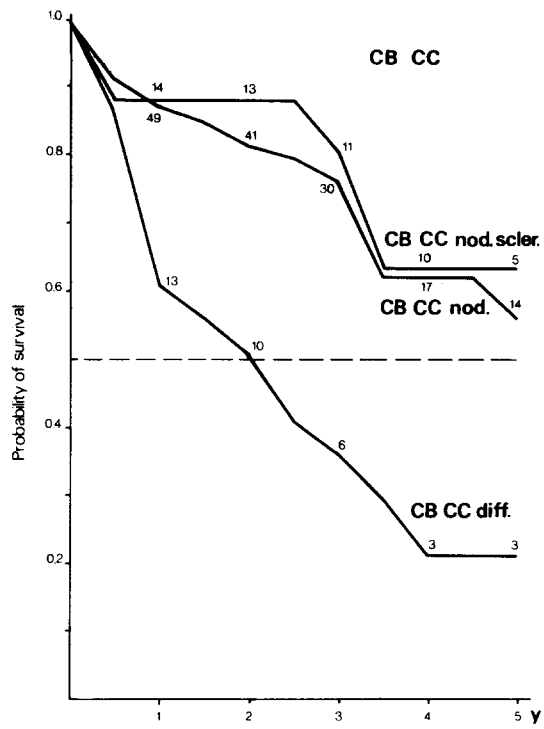

Fig. 5. Crude actuarial survival curves for CB-CC subtypes (nodular, with or without sclerosis, and diffuse).

of the period of treatment; it demonstrates a moderate but regular improvement, in the results between the beginning and the end of the decade in consideration, comparable in the two main histologic groups.

DISCUSSION

The histologic revision of 360 case histories of patients who had initially been diagnosed as having NHML first led to the exclusion of 84 cases; the diagnosis of these cases has been

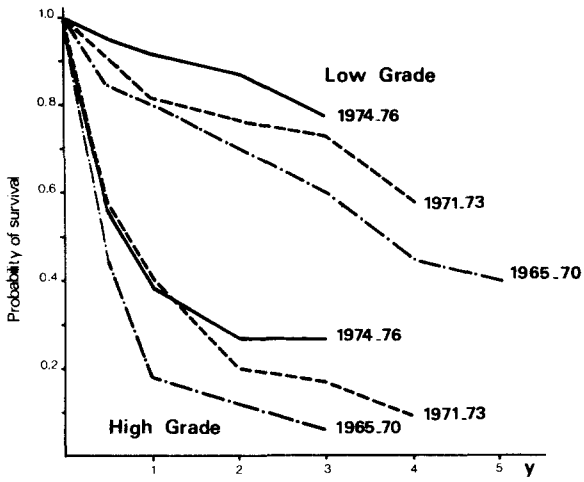

Fig. 6. Crude actuarial survival curves according to the year of diagnosis and first treatment.

modified (Table 1). For forty of them it was really lymph node metastases of undifferenciated carcinoma (anaplastic carcinoma, oat cell carcinoma of the lung, nasopharyngeal carcinoma) or even thymona, melanoma, seminoma, embryonal sarcoma or neuroblastoma. These diagnostic readjustments were largely facilitated during the second reading by the routine use of special stains and, in particular, slow staining Giemsa; this stain only weakly colors metastatic cells, while the cytoplasm of the lymphoid elements are generally bluish or dark blue.

The good technical quality of the sections and the stains is the first condition necessary in order to reduce classification difficulties. Kiel's classification is sufficiently explicit to avoid problems in the large majority of the cases. There may be some hesitations between LC, LP or diffuse CB-CC types, or between LB and IB types; however, there is rarely a hesitation between low grade and high grade forms. Each of these two main groups is characterized by definite cytologic criteria which are based on the size of the cellular elements and their nucleus, the staining characteristics of the cytoplasm, the outline of their nucleus (round, clived, convoluted) the character of their chromatin and the presence or absence of nucleolus, their size and number [3].

The main criticism which can be attributed to our work lies in the fact that the slides were read by only one pathologist. It would have been preferable, of course, to have the slides read by two or three different pathologists.

Our results may be confronted with those previously published concerning the same histologic classification [6–9].

We have thus observed a relatively higher proportion of high grade forms in comparison with most of the other series. The distribution according to sex and age for each histologic subgroup is, on the contrary, comparable in all series. All forms are more frequent in elderly patients. Low grade forms were not observed in children. For the high grade forms, the distribution of the LB is bimodal with two peaks, one around 20 yr and another around 70 yr, while the frequency of the IB increases with age.

It is more difficult to compare the distribution by stages; in other series, there was a pathologic staging according to Ann Arbor Symposium [10], whereas we have used a clinical staging according to Rye Symposium [5]. This fact explains why we have almost as many I and II stages as III and IV stages, whereas in other series stages III and IV clearly predominate.

In all series, the correlation between evolution and histology demonstrates a very significant difference between the low grade and the high grade forms. This difference is more striking in our series than in preceding ones. In all series the CC have a prognosis slightly less favorable than the other low grade forms. The difference, which is especially distinct, between the nodular CB-CC and the diffuse CB-CC is significant in our series. This difference had not previously been observed by the German investigators, but appears here particularly important. In contrast with other data [11], there is no difference in our series between survival of patients with nodular CB-CC, with or without sclerosis. Like Brittinger *et al.* [8], we did not observe a difference between LB and IB, while Musshoff *et al.* [7] observe a more favorable evolution for the LB than for the IB. At last, in all series the survival regularly decreases for the low grade forms, while it has a tendency to stabilize after an initial rapid deterioration for the high grade ones. This tendency which begins to appear in our series is particularly evident in the one of Dühmke and Quäck [9] in which the two curves meet at 10 yr.

As far as we are concerned, this classification gave us a histoprognostic correlation which was much more interesting than the one obtained with Rappaport's classification [2]. It is close to the one independently proposed by Lukes [12] and indicate two very distinct categories of NHML for which a different therapeutic attitude must be proposed. The evolution, very different between these two types of NHML effectively calls for a treatment adapted to the gravity of the disease [13]. Only the subgroup of diffuse CB-CC lymphomas has an intermediary prognosis but it represents less than 10% of the patients (24/274). The group of histologically unclassifiable cases shares the poor prognosis of the high grade forms.

Consequently, our results confirm that Kiel's

classification is useful in indicating the prognosis and guiding the treatment of NHML. The question as to whether these results are reproducible remains unanswered. The convergence of the main series of different teams published until now seems to indicate that this reproducibility is good; however, only studies presently underway involving comparisons between several pathologists will shed more light on this subject.

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Evidence of Pepsinogens in Ovarian Tumors

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Abstract—*Forty-one benign and malignant ovarian tumors and* 12 *normal ovaries were studied immunologically, with extracts of gastric mucosa as reference, in the presence of antimucosa antisera, adult gastric antipepsinogens (anti Pg* I-II, *anti Pg* III *and anti Pg* IV) *and fetal gastric antipepsinogens (anti Pg* IV).

Pepsinogens were identified in 7 *out of* 24 *benign tumors, all of the mucinous type and in* 1 *out of* 17 *malign tumors. Pepsinogen secretion did not occur in normal ovaries.*

The potentiality of pepsinogen synthesis by tumoral ovaries was the same as that of other antigens of the gastric or intestinal type as recently found in the same tumors. This potentiality did not seem to be linked to the malignancy of the tumors studied.

INTRODUCTION

ANTIGENS of the gastric or intestinal type are known to exist in benign and malignant tumors. Tachibana in 1929 and Cariker-Dockerty in 1954 identified "intestinal enzymes" in ovarian tumors. Antigens common to the intestine were also found in ovarian tumors by McNeil *et al.* [1], Nairn *et al.* [2], and Marchand *et al.* [3]. More recently, Bara *et al.* [4] reported common antigenicities between gastric mucoproteins and mucinous ovarian cysts.

According to Evans [5], the histogenesis of ovarian tumors involves a unilateral endodermic development at the expense of multipotential cells capable of giving rise subsequently to the intestinal tract. This known histogenic multipotentiality prompted us to look for gastric pepsinogens in normal and tumor-bearing ovaries. The immunochemical materials and the immunoelectrophoretic and catalytical nomenclature used in our study of pepsinogens has been described previously [6–8].

MATERIAL AND METHODS

Forty-one ovarian tumors of the adenocystic type or solid were studied from material obtained surgically; they included 24 benign tumors and 17 malignant tumors (See Tables 1 and 2).

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Twelve histologically normal ovaries, taken from women 35–75 yr of age were studied concomitantly as controls. (Eight ovaries were from women 35–48 yr of age and 4 ovaries from women 48–75 yr of age.)

(A) *Preparation of the ovarian extracts*

(a) *Tumoral extracts and normal control extracts.* Soluble extracts were prepared by homogenization of the tissues with demineralized water in an Ultraturrax, ultracentrifugation for 20 min at 25,000 rev/min at +4°C and lyophilization of the supernatants.

(b) *Adenocystic secretions.* Intracystic fluids were centrifuged at 25,000 rev/min at +4°C for 20 min and the supernatants were lyophilized. The protein concentrations of the lyophilized extracts, as determined by the biuret reaction, varied between 20 and 40 mg/ml according to their solubility. We used both the secretion fluids and whole-tumor extracts of the 31 tumors of the secreting type. The histological nature of the tumors was studied systematically.

(B) *Reference Material (Gastric pepsinogens)*

*Extracts of gastric mucosa (M*1). These were obtained from the normal looking areas of material removed surgically, according to a procedure described earlier [7]. They contained the 3 pepsinogens which we have defined according to their decreasing electrophoretic mobilities, their antigenicity in the presence of anti-gastric mucosa (normal) antisera, and their optimal proteolytic activities at pH 2.0:

Pg I–II Pg III and Pg IV

Table 1. Non-degenerated ovarian tumors

No.	Name	Tumor type	Secretory type	Pepsinogens Pg I–II, Pg III, Pg IV
1	KI	Enteroid cystoma	mucinous	Pg I–II +, Pg III + + +
2	MAR	Enteroid cystoma	mucinous	Pg III + +, Pg IV +
3	GI	Enteroid cystoma	serous	none
4	BON	Cystic endometriosis	serous	none
5	YN	Dermoid cystoma + enteroid aspect	mucinous	none
6	SCH	Cystic ovaritis	serous	none
7	DAV	Dermoid cystoma	serous	none
8	STE	Cystic endometriosis	serous	none
9	GIA	Cystic ovaritis	mucinous	none
10	OHA	Cystic ovaritis	mucinous	none
11	MEN	Cystic ovaritis	mucinous	none
12	RED	Cystic ovaritis	mucinous	Pg I–II +, Pg III + + + Pg IV traces
13	RE	Dermoid cystoma	serous	none
14	WEC	Enteroid cystoma	mucinous	PG III +
15	MAS	Enteroid cystoma	none	none
16	MAC	Dermoid cystoma	none	none
17	REN	Cystic endometriosis	none	none
18	FOL	Dermoid cystoma	none	none
19	CHA	Enteroid cystoma	mucinous	Pg I–II +, Pg III +, Pg IV traces
20	GLO	Dermoid cystoma	none	none
21	MAT	Dermoid and enteroid cystoma	mucinous	Pg III + +
22	STU	Cystic endometriosis	mucinous	none
23	N	Cystic endometriosis	mucinous	none
24	LAZ	Enteroid cystoma	mucinous	Pg III +

Table 2. Degenerated ovarian tumors

No.	Name	Tumoral type	Secretory type	Pepsinogens Pg I–II, Pg III, Pg IV
1	GU	Malignant dysgerminoma	mucinous	none
2	BAD	Glandular epithelioma (adenocarcinoma)	none	none
3	VEN	Cystadenocarcinoma	serous	none
4	BAC	Germinative epithelioma	none	none
5	PAU	Peritoneal pseudomyxoma + ovarian metastases	mucinous	none
6	PER	Arrhenoblastoma	serous	none
7	CAP	Cystadenocarcinoma	sero-mucinous	none
8	MEJ	Germinative epithelioma	serous	none
9	HAT	Germinative epithelioma with papillary structure	serous	none
10	MAG	Germinative epithelioma	none	none
11	SCH	Spinocellular epithelioma + dermoid cystoma	serous	none
12	COQ	Primary papillary germinative epithelioma	serous	none
13	SEC	Primary papillary germinative epithelioma	none	none
14	LEF	Primary papillary germinative epithelioma	*	Pg I–II + +
15	DUR	Primary papillary germinative epithelioma	none	none
16	N	Primary papillary germinative epithelioma	serous	none
17	A	Primary papillary germinative epithelioma	serous	none

*With benign serous cyst.

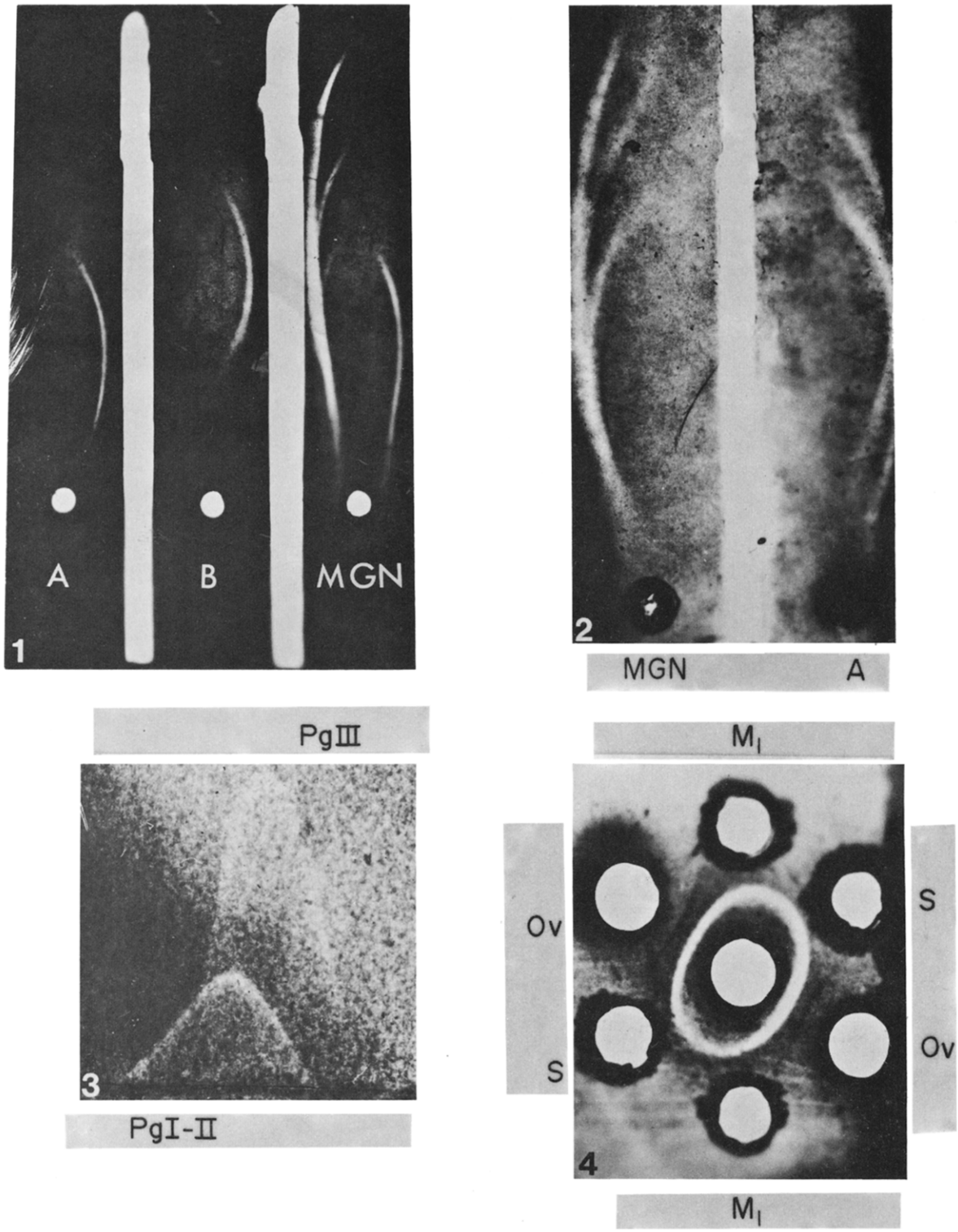

Fig. 1. *Catalytic immunoelectrophoresis of the adult gastric mucosa extract (MGN) fetal gastric extract (A) and an ovarian benign tumor extract (B) After completion of the immunoelectrophoresis the plate is immersed in* 1% *Bovine Serum Albumin at pH* 2.0. *The precipitin lines with proteolytic activity appear clear on a blue background (due to the staining of the undigested protein by Amidoschwarz). Antisera.* 1–*Antiwhole adult gastric mucosa extract (AM).* 2–*Antiwhole fetal stomach extract (AMf) containing only antibodies against pepsinogen Pg* IV.

Fig. 2. *Catalytic immunoelectrophoresis of an ovarian benign tumor extract (A) and of the adult gastric mucosa reference extract (MGN). The proteolytic activities where: A: Pg* III *and Pg* IV; *MGN: Pg* I–II, *Pg* III *and Pg* IV. *Antiserum. antiwhole adult gastric mucosa extract.*

Fig. 3. *Catalytic bidimensional immunoelectrophoresis of an ovarian tumor extract. Two proteolytic activities: Pg* III *and Pg* I–II. *Antiserum. antiwhole adult gastric mucosa extract.*

Fig. 4. *The immunochemical relationship of the Pg* III *pepsinogen in the gastric mucosa* (M_1) *the seminal plasma (S) and in a mucinous ovarian tumor extract (Ov.). Antiserum. antiseminal plasma anti Pg* III.

Antisera. For the anti-human pepsinogen antisera, we used: 1-anti-M1 (adult) antiserum (AM1) obtained against the 3 gastric pepsinogens, 2-anti-fetal gastric mucosa antiserum (AMF) obtained against pepsinogen Pg IV (or fetal Pg) [8], 3-anti-human seminal fluid (ALS) which reacted strongly only against gastric pepsinogen Pg III.

Methods

Standard microimmunoelectrophoresis on gelose (DIFCO) and two-dimensional immunoelectrophoresis were used systematically. The bidimensional migrations were made in 1% agarose (Indubiose IBF, Gennevilliers, France) and in a 0.02 M veronal buffer at pH 8.2. For the first dimension, migration occurred for 2 hr under a continuous current of 30 mA and 6V/cm at +4°C. Migration during the second run was for 18 hr in a gel containing 2 and 3 ml of antiserum (according to the experiment) and under 2.5 mA and 2V/cm at +4°C.

The plaques were examined for proteolytic activity of the pepsin type after they were washed (24 H) in buffered physiological saline at pH 7.4 and dried. The films of gel were incubated in a substrate of 1.0% bovine albumin serum (ICN Biochemicals, Cleveland, U.S.A.) in 0.2 M HCl-glycine buffer at pH 2.0. After 30 min at room temperature, the samples were incubated at 37°C for 30 min and fixed in 5% acetic alcohol for 30 min. The proteolytic activities were observed after coloration with Amidoschwarz (hydrolysis bands were clear), Figs. 1–3.

RESULTS

The results are summarized in Tables 1 and 2 and in the Figs. The proteolytic activities of pepsin and the immunochemical identification of the pepsinogens were noted in 8 of the 41 ovarian tumor extracts studied. Seven of these cases were non-degenerated tumors of the mucinous type. The 8th case was a primary papillary germinative epithelioma.

Identification of the pepsinogens in the secreting tumors was more frequent in the secretions than in extracts prepared from the solid tumoral part: of the 7 cases with positive cysts, pepsinogens were identified only twice in extracts from the solid material.

In the 12 ovarian extracts of the normal controls, as well as in the secreting tumors of the serous type, no precipitin bands of pepsinogen or pepsin activity were detected.

Studies by double immunodiffusion (Ouchterlony) allowed us to observe the antigenic similarities first between the gastric pepsinogens and seminal fluid and then between the ovarian constituents. (Fig. 4).

Nature of the pepsinogens identified

The 3 pepsinogens of the normal gastric mucosa were present simultaneously only in 2 cases of non-degenerated tumors: 1 enteroid cyst and 1 dermoid cyst that were partially mucoid. Two other cysts contained 2 pepsinogens at the same time. In the 4 other cases, only a single pepsinogen was evident. Pepsinogen Pg III was found most often (7 out of 8 times). Pg I–II was found 4 times and Pg IV 3 times. The degenerated tumor contained only Pg I–II (see above).

DISCUSSION

From the above results it appears that ovarian tumors are at times capable of synthesizing pepsinogens. These pepsinogens are identical with those of the gastric mucosa, as observed from their electrophoretic and catalytic immunological characteristics [6, 7]. Three facts should be emphasized:

1. The identification of the pepsinogens was most frequent in non-degenerated ovarian tumors: in 7 out of 24 non-degenerated tumors and only 1 out of the 17 ovarian cancers studied. It is also possible that only the benign part of the cancer produced the pepsinogens. (Fig. 3). All the non-degenerated tumors containing the pepsinogens were of the mucinous cystic type.

2. Identification of pepsinogen Pg III was particularly frequent (7 out of 8 times). Pepsinogen Pg IV, which we identified earlier as the principal fetal pepsinogen [8], was identified only 3 times. In the gastric cancers and in the gastric metaplasia of the intestinal type studied earlier, the same fetal pepsinogen was constantly present while pepsinogens Pg I–II and Pg III were decreased or absent.

3. A seminal pepsinogen was reported earlier by Lundquist and Seedorf in seminal fluid [9]. We found it to be identical with Pg III of the gastric mucosa [10]. In this work, we were able to determine the immunochemical identity of the Pg III pepsinogens of the gastric mucosa, seminal fluid and ovary. It should be noted that Pg III did not exist in the normal ovaries studied. As for the seminal fluid, it has not been possible to explain the role and the mecanism of appearance of a precursor of pepsin (active at very acid pH) in the alkaline media which represent the cystic secretions of ovarian origin. Our

studies on the synthesis by the tumoral ovary of constituents having a gastric antigenicity may be compared to the work of J. Bara *et al.* [4] who identified high molecular weight glycoproteins that were common to the normal gastric mucosa in mucinous cysts of the ovary.

Similarities between the antigenic groups of the gastro-intestinal tract and the tumoral ovary was shown by McNeil *et al.* [1] and Stone *et al.* [11] who detected carcinoembryonic antigens (CEA) in cancers of the ovary. Nairn *et al.* [2] identified antigens of the intestinal type in sections of cystic adenomas using immunofluorescence. This work was confirmed by Marchand *et al.* who identified CEA in the ovarian adenocarcinomas of the mucinous but nor serous type [3].

More recently, Bara *et al.* [4] identified a sulfoglycopeptide normally associated with the caliciform cells of the colic mucosa, in 5 mucinous tumors of the ovary.

In our patients, the appearance of pepsinogens in the tumoral ovary seemed less frequent than the findings of CEA by others in tumors of the ovary. Moreover, the presence of pepsinogens does not appear to be linked to the idea of malignancy, since their frequency was much greater in non-degenerated tumors.

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Letters to the Editor

Glucose Metabolism in British and Japanese Breast Carcinomas

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THE INCIDENCE of breast cancer in Japan is significantly lower than in Western countries [1, 2] and, furthermore, the survival rate in Japanese patients is better than in their Western counterparts [3, 4]. Attempts have been made to explain these differences on the basis of endocrine status as measured by concentrations of hormones and their metabolites in body fluids [5, 6]. However, no major differences were observed between the two populations. As far as Japanese cancer patients are concerned, the urinary titres of androgen metabolites, the amounts of oestrogen receptor in the neoplasm and responsiveness to endocrine treatment is similar to that in British or American patients [7, 8].

Despite similarities in endocrine status, there are certain histopathological differences between Japanese and Western tumours in that the incidence of medullary carcinomas with lymphoid stroma is significantly higher in Japanese patients [9] and there is more intense infiltration of other carcinomas by plasma or lymphoid cells [4, 10, 11]. This, and the higher frequency of sinus histiocytosis noted in the axillary lymph nodes of Japanese patients [10, 11] has led to the postulate that both transformation of the normal cell to incipient carcinoma and of incipient to invasive carcinoma may be related to host resistance which results in the differences observed in prognosis. Whether these pathological differences are reflected in alterations in biochemical processes such as glycolysis, is not known and a comparative study between carcinomas from Japanese and Western patients might give further insight into the findings. Therefore, an attempt was made to estimate the activities of certain enzymes in Japanese carcinomas and to compare them with tumours from British patients.

The details of enzymes, co-enzymes and substrates used in the study are described in detail elsewhere [12]. Primary breast carcinomas were obtained from 22 Japanese and 49 British women undergoing mastectomy. The Japanese tumours were kept on cardice during transport to Britain and remained frozen until further processing. The malignancy of tumours was confirmed by histological examination. The tissues were cleared of any surrounding fat, weighed and cut into small pieces. The semi-frozen tissues were then homogenised in a Silverson homogeniser according to the procedure of Shonk and Boxer [13]. The homogenates were centrifuged (800 ***g*** for 15 min) at 4°C, the cytosol fraction was decanted off and used as a source of enzymes. A 5% solution of tumours was used in these estimations. The activities of α-glycerol-phosphate dehydrogenase (α-GPDH), glucose-6-phosphate dehydrogenase (G6PDH), phosphofructokinase (PFK), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucomutase (PGM), lactate dehydrogenase (LDH) and phosphohexose isomerase (PHI) were measured. Enzyme assays were performed in a Beckman recording spectrophotometer according to the procedure of Shonk and Boxer [13], with the exception that one minute readings were recorded automatically for 5 min and a mean of the last four was taken as an average change in optical density. Deoxyribonucleic acid (DNA) was estimated according to the procedure of Burton [14]. The results were calculated on the basis of units (U) of enzyme activity per milligram of DNA. A unit is defined as that amount of enzyme which will catalyse the transformation of 1 μmole

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of substrate per minute. The results are presented as mean ± S.D. (Table 1). The means were tested for significance by a Student's *t*-test.

Table 1. *Enzyme activities* (μmole *of pyridine nucleotide reduced or oxidised*/min/mg *of DNA*) *in British and Japanese tumours*

Enzyme	U/mg DNA British	Japanese
PFK	0.033 ± 0.019*	0.101 ± 0.073
G6PDH	0.077 ± 0.042*	0.281 ± 0.213
6PGDH	0.084 ± 0.048†	0.046 ± 0.021
PGM	0.892 ± 0.542	0.871 ± 0.543
LDH	3.820 ± 1.930	4.017 ± 2.261
α-GPDH	0.168 ± 0.116*	0.079 ± 0.062
PHI	4.955 ± 2.481	5.380 ± 2.986

They are expressed as mean ± S.D.
*Significant differences between the two groups $P < 0.001$.
†Significant differences between the two groups $P < 0.01$.

Our results from this pilot study show that there are certain similarities between enzyme activities in breast carcinomas from Japanese and British patients. The activities of PGM, LDH and PHI were similar in the two groups. Assuming that results expressed in terms of DNA measurements cancel out any cellularity differences between two groups of tumours, it appears that the rates of glycogen breakdown and lactate production are similar in both groups. In contrast, significantly higher PFK and G6PDH and lower 6PGDH and α-GPDH activities were observed in the Japanese tumours. The higher activities of PFK in these tumours could indicate increased activity of the whole glycolytic sequence. Whereas lower α-GPDH activities suggest decreased exchange of substrates between lipid and carbohydrate metabolism at this point. Whether these differences in enzyme activities correlate with either prognosis or the histopathological differences mentioned in the introduction remains to be explored.

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Letters to the Editor

Improvement of Therapeutic Effects of Cytosine-Arabinoside on Walker-Carcinosarcoma after Different Synchronization with Hydroxyurea

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VARIOUS studies have reported that the therapeutic results of various cytostatic agents are improved after partial synchronization of animal and human tumours [1, 2]. Some authors have questioned, however, whether these improved therapeutic results are really due to a partial synchronization of the tumour cells or whether the results might not be explained by the combination therapy with a particularly favourable timing for the application of the substances [3, 4]. In earlier experiments we demonstrated that drugs with activity for specific phases of the cell cycle gave better therapeutic results if they were given at a certain phase of the cell cycle. Thus cytosine arabinoside (Ara-C), hydroxyurea (HU) and vincristine (VCR) produced better therapeutic effects on the Walker-carcinosarcoma after partial synchronization (with HU) if these drugs were given at the G_1/S border or S-phase [5].

To test if these improved therapeutic results are really due to a partial synchronization of tumour cells, we chose for synchronization two different experimental models:

1. Application of 6 × 50 mg/kg HU (Fig. 1 left, top) [5]. The blockage at the G_1/S border lasted 8.5 hr and ended 1.5 hr after the last application of HU.
2. Application of 1 × 300 mg/kg HU (Fig. 1 right, top). The blockage lasted 4 hr.

At specific times after the last HU-application the synchronized and non-synchronized animals with solid Walker-carcinosarcoma (transplanted s.c. in the skin of the back 4 days before synchronization) were treated with 1 × 400 mg/kg Ara-C i.p.

The tumour size was measured between the 1st and 3rd day after treatment using a movable gauge and its daily growth was determined (Fig. 1 bottom). As is evident, the effects of therapy with Ara-C can be related to the single phases of the cell cycle. With the two different procedures of synchronization the clearest effect of therapy is obtained at the G_1/S border. As compared with the non-synchronized animals, no better effects of therapy on the synchronized animals can be demonstrated in the G_2-phase and M-phase. These results agree with those of Persohn *et al.* [6] who could demonstrate with a synchronized human lymphoma cell line that the uptake of radioactive Ara-C is greatest during the early S-phase and declines steadily.

The results of these experiments indicate that the improved therapeutic effects of Ara-C are in fact due to the synchronization of the tumour cells.

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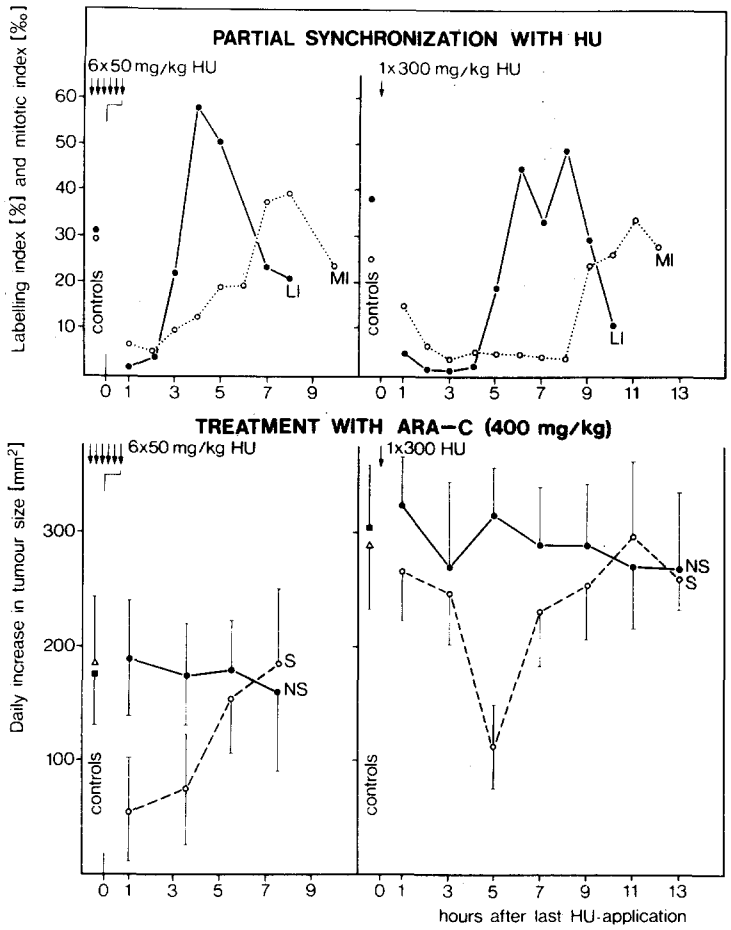

Fig. 1. Top. Effect of 6 × 50 mg/kg *HU (left, average values from 7 animals at each point,* n = 70 *rats) and* 1 × 300 mg/kg *HU (right, average values from 4 animals,* n = 52 *rats) on solid Walker carcinosarcoma (Sprague–Dawley rats, ♂, 200 g) on labelling index (LI,* ●——● *) and mitotic index (MI,* ○ . . . ○*), controls (not treated with HU).*

Bottom. Daily increase in tumour size of the solid Walker carcinosarcoma after treatment with 400 mg/kg *Ara-C at different time points after partial synchronization with HU. Left: synchronization with* 6 × 50 mg/kg *HU, average values from* 10 *animals at each point,* n = 100 *rats; right: synchronization with* 1 × 300 mg/kg *HU, average values from 7 animals,* n = 112 *rats.*

±; *standard deviation.*
NS, ●——●; *treated with Ara-C, non synchronized.*
S, ○– – –○; *treated with Ara-C, synchronized.*
■; *non treated with Ara-C, non synchronized.*
△; *non treated with Ara-C, synchronized.*

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Announcement

"The 12th International Leukocyte Culture Conference" will be held at the Ben Gurion University of the Negev, Beersheba, Israel, on 25–30 June, 1978.

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T. Nagayo and H. Yokoyama: Recent changes in the morphology of gastric cancer in Japan.

J. P. Harlos and L. Weiss: Comparison between the macrophage electrophoretic mobility (MEM) and the fixed tanned erythrocyte electrophoretic mobility (FTEEM) tests in the detection of cancer.

G. Johannesson, G. Geirsson and N. Day: The effect of mass-screening in Iceland 1965–1974 on incidence and mortality of cervical carcinoma.

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P. G. Parsons and L. Morrison: Melphalan-induced chromosome damage in sensitive and resistant human melanoma cell lines.

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Tumor Associated Lymphocyte Cytotoxicity Correlated with Stage of Disease in Patients with Colon Adenocarcinoma

*A Preliminary Report**

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Abstract—*Whole lymphocyte populations and T and non-T subpopulations from 22 colorectal carcinoma patients (8 preoperative; 6 postoperative disease free; 8 postoperative residual disease) and 6 control Stage III melanoma patients were tested in parallel on a colon cell line (P-4788) and a melanoma cell line (M-7292). All three effector populations from the four patient groups were cytotoxic on both cell lines. Within the different donor groups, the unfractionated lymphocytes from preoperative colon patients displayed significantly greater cytotoxicity than the Stage III melanoma patients on the colon cell line. Conversely, the Stage III melanoma patients displayed significantly greater cytotoxicity than the postoperative disease free colon patients on the melanoma cell line. Furthermore, the unfractionated lymphocytes and non-T subfraction from preoperative and postoperative disease free colon carcinoma patients showed significantly stronger cytotoxic effects on the colon cell line than on the melanoma cell line. These two lines of evidence suggest that both tumor associated cytotoxicity and spontaneous or non-selective cytotoxicity are present; and the presence of tumor associated cytotoxicity is dependent on the stage of the disease. Furthermore, thr level of cytotoxicity associated with both postoperative colon carcinoma groups was lower than the preoperative colon carcinoma groups. However, this depression was not evident in subfractions of lymphocytes that demonstrated apparent tumor associated cytotoxicity and thus may reflect a decline in general immune competence.*

INTRODUCTION

CELL-MEDIATED immunity to human tumors is estimated by the *in vitro* lymphocytotoxicity assay [1–3]. In recent years, however, extensive testing has revealed cytotoxic reactivity by normal individuals [4] and lack of histological type specific reactivity in cancer patients (spontaneous or non-selective cytotoxicity) [5]. Furthermore, selective enrichment of T and non-T subpopulations of lymphocytes by rosetting techniques have indicated that T-cell peripheral blood lymphocyte enriched fractions are more cytotoxic than T-cell depleted fractions from patients with melanoma [6] and colon carcinoma [7]. However, in another recent melanoma study, cytotoxic reactivity was found to be associated with the non-T cell fraction [8].

These contradictory findings imply that still another factor with regard to differential cytotoxicity may be involved. These studies have not adequately looked at the effect of disease status as an added factor of variability on associated cytotoxicity. Different tumor bearing states or the stage of disease may dictate the relative contribution of different effector cell mechanisms. This has been demonstrated with Maloney sarcoma virus induced tumors [9, 10]. Consequently, a study was undertaken to see if differences in cytotoxicity could be related to disease status, particularly with regard to selectively enriched lymphocyte subpopulations in patients with colon adenocarcinoma.

MATERIAL AND METHODS

Colon patients

Twenty-two colorectal carcinoma patients were tested. The ages ranged from 40 to 81 yr with

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an average of 65 yr. Eight Dukes D patients were tested preoperatively. Two postoperative groups included 6 patients with no evidence of disease (Dukes B and C) and 8 patients with residual metastatic disease (Dukes D). All postoperative patients in this study were evaluated at least two weeks post-surgery.

Melanoma patients

Six Stage III melanoma patients were used as controls for histological specificity. These patients were receiving chemotherapy for metastatic disease but were tested prior to treatment.

Target cells

RPMI-4788, an established colon carcinoma cell line, was used as the principle target cell: HCT-8, an established colon carcinoma cell line and RPMI-7292 (M-4), an established amelanotic cell line, were used as indicators of histological specificity. All cells were propagated as monolayer cultures in RPMI-1640 with 15% fetal calf serum (FCS) with penicillin and streptomycin. Cells were harvested for testing with 0.25% trypsin and 0.25% EDTA.

Lymphocyte preparation

Lymphocytes were separated from EDTA-anticoagulated whole blood on a double discontinuous Ficoll–Hypaque gradient with modifications to selectively remove platelets and monocytes [11]. All cell populations used had greater than 95% viability as assessed by trypan blue and consisted of greater than 94% lymphocytes as assessed by Wright's stained differential of cytocentrifuge cell preparations.

T and non-T cell preparation

T-cell rosettes were formed as follows: 1 ml of 1% sheep red blood cells (SRBC) washed (×5) in normal saline was added to 1×10^7 lymphocytes and these suspensions were centrifuged at 200 ***g*** for 3 min. The resulting pellets were refrigerated at 4°C, usually overnight, to allow (T) rosettes to form. Pellets were disrupted by gentle Pasteur pipetting and the number of (E) rosettes was quantitated by microscopic counting. Counts averaged between 40 and 60% (E) rosettes. The resulting T and non-T cells were separated by layering an aliquot of the rosette cell suspension on 3 ml of Ficoll–Hypaque (s. g. 1077) and spinning at 400 ***g*** for 20 min. Non-rosetted cells stay at the interface and rosetted cells pellet. All three subsequent cell populations, whole, T and non-T were treated with Boyle's solution for 5 min to lyse SRBC's and then were pelleted and washed twice in RPMI-1640. Homogeneity of effector cell populations was monitored by immunofluorescence and T-cell rosetting. By these two criterion, the rosetted fractions (T-enriched) contained approximately 80% T-cells while non-T fractions (T-depleted) contained 50–60% B cells with less than 10% contaminating T cells.

Microcytotoxicity test

The microcytotoxicity test was performed as described by Takasugi and Klein [12]. The lymphocytes were tested in ratios of 100, 75 and 50 per target cell and each was tested in six replicates. Residual target cells attached to the bottom of the test wells were enumerated by a semi-automated technique devised by this group [13]. In this assay, mean percent cytotoxicity is 1 minus the ratio of the mean number of target cells in the presence of lymphocytes to the mean number of target cells in the absence of lymphocytes (medium control). To simplify the presentation of data, the results of Table 1 are presented as a mean target cell reduction calculated according to the method of Takasugi *et al.* [5] utilizing the following formula:

$$\text{Mean } \% \text{ reduction} = \frac{A+B+C}{3}$$

Where

A = Mean % cytotoxicity at E : T ratio 100 : 1
B = Mean % cytotoxicity at E : T ratio 75 : 1
C = Mean % cytotoxicity at E : T ratio 50 : 1

The Mann–Whitney U-Test was used to compare experimental groups. A P value of less than 0.05 was considered significant.

RESULTS

Lymphocytes (whole, T and non-T) from colorectal patients and melanoma patients showed cytotoxic effects of all three effector to target cell ratios and on both target cell lines. The cytotoxicity observed with both T- and non-T effector cells was similar to the cytotoxicity observed with the whole lymphocyte population. No significant observations were obtained when the cytotoxic effects of whole, T- and non-T lymphocytes from colorectal and melanoma patients were compared. However, when the colorectal patients were divided into subgroups on the basis of disease status, significant observations could be made between different subgroup effector populations on the same cell line, as well as between the same subgroup effector population on different cell lines.

In a comparison of different subgroup effector populations on the same cell line (Table 1),

Table 1. Mean percentage of cytotoxic effects of lymphocytes from colon carcinoma and malignant melanoma patients on established colon and melanoma cell lines

Whole effector cells	Colon	P*	Melanoma
Colon carcinoma patients			
Preoperative	90.2	0.05	67.6
Postop. disease free	65.4	0.05	51.1
Postop. residual disease	64.1	NS	57.6
Melanoma patients stage III	66.6	NS	66.3
Non-T effector cells			
Colon carcinoma patients			
Preoperative	72.2	0.05	59.3
Postop. disease free	65.2	0.05	47.6
Postop. residual disease	49.2	NS	55.0
Melanoma patients Stage III	68.8	NS	60.0
T effector cells			
Colon carcinoma patients			
Preoperative	80.6	NS	81.8
Postop. disease free	49.4	NS	45.1
Postop. residual disease	48.5	NS	55.8
Melanoma patients Stage III	58.0	NS	42.5

* Statistical comparison of cytolytic ability of the same effector cells on histologically distinct target cells. Comparison between effector cells on the same target cell is presented in the text.

preoperative colon carcinoma patients were significantly more reactive than Stage III melanoma patients on the colon cell line ($P < 0.05$). In contrast, these two groups of patients displayed the same degree of cytolysis on the melanoma cell line. Furthermore, the Stage III melanoma patients were significantly more reactive than the postoperative disease free group on the melanoma cell line ($P < 0.05$). In contrast, these two groups gave the same degree of cytolysis on the colon cell line.

In a comparison of the same subgroup effector population on different cell lines, the whole effector cells from both the preoperative and postoperative no evidence of disease group were more cytotoxic on the colon cell line than the melanoma cell line ($P < 0.05$). In contrast, the same degree of cytotoxicity was observed on both cell lines with the postoperative residual disease group and the Stage III melanoma group. Furthermore, this pattern of cytolytic activity was mirrored by the non-T cell subpopulation And not by the T-cell subpopulation.

In addition, it was observed that the whole effector cells from the preoperative group were more cytotoxic than either of the postoperative groups (Table 2). This difference was most pronounced in the T-cell subpopulation, but was also observed with the non-T effector cells from the postoperative residual disease group.

Table 2. Degree of reduction in percentage of cytotoxicity preoperative vs postoperative†*

Clinical status	Effector cells		
	Whole	T	Non-T
Postoperative residual disease	28.9%	39.8%	31.8%
	$P < 0.05$	$P < 0.01$	$P < 0.05$
Postoperative disease free	27.5%	38.7%	9.7%
	$P < 0.05$	$P < 0.01$	Ns

* $\frac{\text{Reduction preoperative} - \% \text{ reduction postoperative}}{\% \text{ Reduction preoperative}}$.

† On colon carcinoma target cells.

DISCUSSION

The purpose of this study was to evaluate disease status as a factor influencing tumor associated cytotoxicity. Initially, the cytotlytic ability of effector cells from normals, colon patients and melanoma patients was evaluated on two established colon adenocarcinoma cell lines (P-4788, HCT-8) and one control melanoma cell line. The cytolytic ability of normal effector cells from various age groups varied considerably [14] and, thus, precluded their use as a control group. As a similar pattern of cytotoxicity was observed on both HCT-8 and P-4788 cell lines, the HCT-8 cell line was dropped in favor of testing a larger group of patients.

No significant results were observed when the cytotoxic effects of lymphocytes from colorectal and melanoma patients were compared. However, by comparing the cytolytic ability of the different patient subgroups (preoperative, postoperative disease free and postoperative residual disease) on the same cell line and the same patient subgroup on the two histologically distinct cell lines, it appeared that both tumor associated cytotoxicity, as well as spontaneous or non-selective cytotoxicity could be detected. Thus, in trying to assess the presence of tumor associated cytotoxicity, it is important not only to take into account the type of disease the patient has, but also the stage of the disease.

A similar pattern of differentially stronger cytotoxicity was observed with lymphocytes from Stages I and II melanoma patients on a melanoma cell line, in comparison to a bladder carcinoma cell line [15]. Thus, both studies are in accordance with the concept of tumor associated cytotoxicity superimposed on spontaneous cytotoxicity. Furthermore, it is evident from this study that both T- and non-T effector cells demonstrated spontaneous cytotoxicity. However, tumor associated cytotoxicity appeared to be associated with the non-T effector subpopulation. Contradictory findings about subpopulation lymphoid activity have been reported in the literature. O'Toole *et al.* [16] and de Vries *et al.* [8] reported that tumor associated cytotoxic activity could be recovered in the non-T effector population with bladder carcinoma and malignant melanoma patients, respectively. In contrast, Wybran *et al.* [6] and Nind *et al.* [7] reported that the tumor associated cytotoxic activity could be recovered in the effector subpopulation. It may be noted that in the former group, rosetted cells were treated with Boyle's solution to lyse the sheep red blood cells, whereas in the latter group, the rosetted fraction was not treated with Boyle's solution, but sheep red blood cells were added to the non-rosetted fraction to obtain the same cell density. Any effects of ammonium chloride treatment on cell-mediated immune reactivity are not well characterized at present.

Whole, T- and non-T effector cells from postoperative colon carcinoma patients with residual disease and Stage III melanoma patients displayed the same degree of cytolysis on the two cell lines. The cell-mediated antitumor immune response of Stage III melanoma patients has been previously characterized. De Vries *et al.* [15] reported that lymphocytes from Stage III melanoma patients displayed the same degree of cytotoxicity on the melanoma cell line and on a control bladder carcinoma cell line. Furthermore, Marti and Thomson [17] demonstrated a markedly decreased response by Stage III melanoma patients in a leukocyte adherence inhibition test in comparison to Stage I melanoma patients. Thus, our observations are in accordance with the above studies.

Colon adenocarcinoma patients were monitored before and after surgery. After surgery, there was a reduction in the level of cytolysis, both with the lymphocytes from patients with residual disease, as well as from patients who were clinically free of disease.

Several studies [18–20] have reported on a depression of the cell-mediated immune response following surgery. However, their studies were conducted one to two weeks after surgery. The patients included in this study were evaluated a minimum of two weeks after surgery. Thus, surgery may have a long-term immunosuppressive effect. However, it is also important to note that the greatest immunosuppressive effect was associated with the subpopulation of effector cells that did not appear to demonstrate tumor associated cytotoxicity. Consequently, surgery may depress the patients' general immune competence, but not the patients' ability to generate a tumor specific immune response. While this data is suggestive of tumor associated cytotoxic reactivity being present during some but not all stages of colon carcinoma, it is imperative that a larger study be carried out with additional patients and a larger number of cell lines so that definitive statements with regards to tumor specific reactions can be made. Furthermore, this study should evaluate the cytolytic effects of normal healthy volunteers so that spontaneous or non-selective cytotoxicity will be defined both as the lack of histological type specific reactivity in cancer patients, as well as the cytotoxic effects of normal donors. This information is necessary to interpret the observed decline in the cell-mediated immune response following surgery.

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Effect of Antiestrogen Therapy on Human Mammary Carcinomas with Different Estrogen Receptor Contents*

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Abstract—*Sucrose gradient centrifugation analysis was used to measure estradiol receptor levels in 35 patients with inoperable or recurrent mammary carcinomas that were subsequently treated with the antiestrogen tamoxifen (Nolvadex ICI). Carcinomas from eighteen patients demonstrated specific estradiol binding. Twelve of these patients responded to therapy. Seventeen patients had tumors without measurable estradiol binding. One of these responded to therapy. Tumors binding less than approximately* 0.1 fmole *of estradiol per* μg *DNA demonstrated a low response rate to tamoxifen* (1/26).

Electrofocusing of the estradiol receptor at pH 6.6 *in polyacrylamide gel was found to be a quantitative and very sensitive method for the measurement of the estradiol receptor content in mammary carcinoma. Sixteen patients had their tumors analyzed with electrofocusing and all demonstrated some specific estradiol binding. The amount of estradiol binding in tumors from responders to tamoxifen or ovariectomy was approximately* 20 *times greater than the amount bound in tumors from non-responders.*

We conclude that there exists a positive correlation between the amount of specific estradiol binding in human mammary carcinomas and their response to subsequent antiestrogen therapy.

INTRODUCTION

SEVERAL lines of evidence have shown that many human mammary carcinomas are estrogen dependent. (1) Many breast cancers contain specific estrogen receptors [1]. (2) About one third of the human mammary carcinomas respond to alteration in estrogen concentration induced by oophorectomy, adrenalectomy or additive estrogen therapy [1]. (3) A strong correlation has been established between estrogen receptor content and the response to these types of therapy [1]. (4) Human breast cancer cells replicating *in vitro* may be stimulated by estrogen or inhibited by antiestrogen [2].

A positive correlation has been demonstrated between the amount of estradiol binding in mammary carcinomas and their probability of response to various types of endocrine therapy [3].

This report purports to explore the correlation between the quantity of estrogen receptors and the response to antiestrogen therapy. Moreover, we will give some further information on the correlation between the response to ovariectomy and the quantity of estrogen receptors.

MATERIAL AND METHODS

Patients and tumours

Fifty-one tumor samples from 43 women with advanced mammary carcinoma were analyzed prior to therapy. The samples were obtained from eleven inoperable breast cancers, 27 subcutaneous metastases and five lymph node metastases.

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Therapy and evaluation

Thirty-five postmenopausal women received 20 mg orally twice daily of the antiestrogen tamoxifen (ICI-46 474; NSC-180 973). Eight premenopausal women were treated by oophorectomy.

Only patients with measurable soft tissue disease were included in the study. The criteria for evaluation were previously described [4] and these specify that remission had occurred when the sum of the products of the two largest tumor diameters had decreased by 50 %.

Measurement of estrogen receptor

Surgical biopsies were provided by two of us (POG) and (NOT) and immediately transferred to the laboratory in icecold saline. They were trimmed of fat and normal tissue and analyzed immediately or frozen at −70°C. The incubation of fresh or quickly thawed carcinomas with ^{3}H-estradiol and the receptor quantification using sucrose gradient centrifugation were previously described [6].

Recently we have improved the electrofocusing procedure for measurement of estradiol binding in human mammary carcinomas [5]. The method is based on the focusing of the partially trypsinized estradiol receptor in polyacrylamide gel at pH 6.6. It is more specific and more sensitive as compared to sucrose gradient analysis and it is not dependent on parallel incubations with excess ligand [5]. Comparison of twenty-five mammary carcinomas that were receptor positive with both sucrose gradient analysis and electrofocusing assay (not illustrated) yielded an average of 2.1 times higher specific estradiol binding values as compared to sucrose gradient analysis. These higher values are probably explained by the facts that the electrofocusing is much more rapid and minimises ligand dissociation and that all receptor material is quantitatively recovered in a sharp peak of radioactivity [5].

RESULTS

Sucrose gradient analysis and results of endocrine therapy

Figure 1 compares specific estrogen binding as measured by sucrose gradient analysis and results of therapy. The binding varied between 0.03 and 3.3 fmole/μg DNA.

Twenty-two patients did not respond to tamoxifen. Six of them had tumors with detectable estradiol binding and one non-responder demonstrated a high binding capacity of 0.7 fmole/μg DNA. Thirteen patients responded to therapy (6 complete and 7 incomplete responders). Twelve of these 13 patients had "receptor-positive" tumors while, in 1 tumor, we were unable to measure the binding. The amount of estradiol binding in the "positive" tumors from responders to tamoxifen varied within wide limits (0.16–3.3 fmole/μg DNA) and there was no obvious difference in binding between complete responders and incomplete responders respectively.

Previous results with patients treated with ovariectomy have demonstrated that receptor-negative patients infrequently respond to endocrine therapy. Our data in Fig. 1 are in accordance with that interpretation.

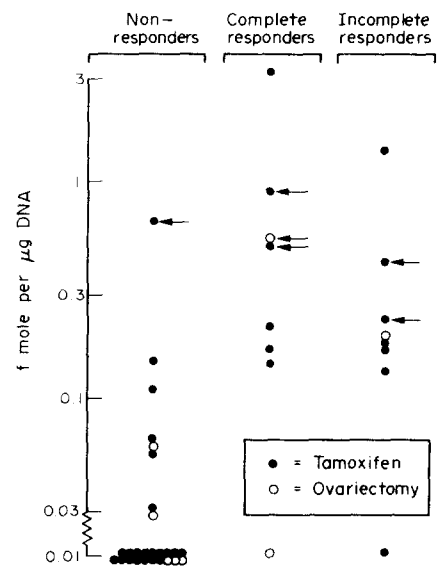

Fig. 1. Estradiol binding as determined by sucrose gradient analysis and response of therapy. Biopsies were removed from mammary carcinomas subsequently treated with tamoxifen (●) or ovariectomy (○). Estradiol binding for each patient in three different response categories is indicated. Arrows indicate tumors with estrogen receptor sedimenting as an 8S complex.

Electrofocusing analysis and results of endocrine therapy

Figure 2 compares specific estradiol binding, as measured by electrofocusing and results of treatment with tamoxifen or ovariectomy in 16 patients.

Tumors from all 16 patients demonstrated some binding. The non-responders bound 0.01–0.23 fmole/μg DNA while tumors from seven responders bound 0.07–1.0 fmole/μg DNA.

DISCUSSION

The antiestrogens bind to the cytoplasmic estrogen receptor in targent cells and are probably transported into the nucleus [7]. The

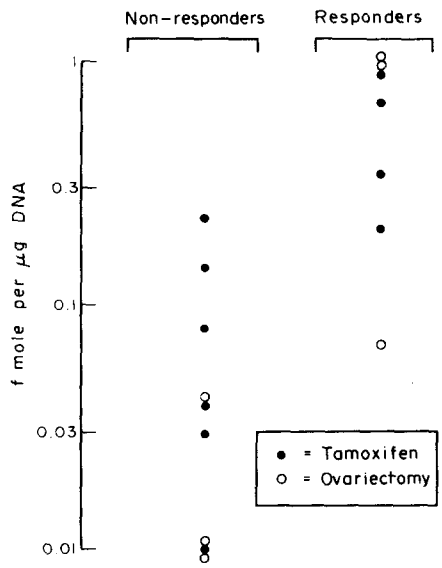

Fig. 2. Estradiol binding as determined by electrofocusing and response to tamoxifen or ovariectomy.

exact intranuclear mechanism of action remains unclear but it is obvious that binding to the estrogen receptor is necessary for biologic action of antiestrogen.

Recent reports indicate that response to antiestrogen therapy only occurs in tumors with estrogen receptors [8].

With several types of endocrine therapy most investigators observed an increased response rate among receptor positive carcinomas [1]. Some investigators using sensitive technique that classified approximately 80% of the mammary carcinomas as "receptor-positive" did, however, report a poor response rate among positive tumors [9–10]. It was therefore suggested that the quantity of receptor per unit tissue should be taken into account and that the labelling of all tumors with specific estradiol binding as "receptor-positive" is not meaningful [9–10].

When this work was initiated we considered sucrose gradient analysis to be most adequate for quantitative analysis. Using this technique we found that 12 out of 13 tumors from responders to tamoxifen bound 0.16–3.3 fmole estradiol per μg DNA while two out of 22 non-responders demonstrated specific binding within this range. This difference is highly significant ($P<0.1\%$). We therefore interpret the data in Fig. 1 to mean a strong correlation between estradiol binding in mammary carcinomas and their response to tamoxifen therapy. Estradiol binding below 0.1 fmole/μg DNA as measured by sucrose gradient analysis does not appear to be associated with an increased response rate to tamoxifen. Two patients were marked exceptions to this pattern. One incomplete responder had a negative tumor and one non-responder had a tumor with significant binding. There are several possible explanations to these exceptions including receptor inactivation in the non-binding tumour or inadequate selfmedication by the non-responder with a strongly binding tumor.

The electrofocusing method used in recent analysis is more sensitive than sucrose gradient analysis and detected some specific binding in all sixteen patients illustrated in Fig. 2. Most tumors from non-responders demonstrated only small amounts of receptor focusing in the pH 6.6 region and the median binding with the non-responders was 0.04 fmole/μg DNA as compared to a median of 0.8 fmole for responders.

Electrofocusing yields average specific estradiol binding values about twice as high as sucrose gradient analysis. We therefore interpret our data in Figs. 1 and 2 to mean that estradiol binding less than 0.2 fmole/μg DNA as measured by electrofocusing is not associated with a clinically significant response rate to tamoxifen. It should, however, be realized that estradiol binding data should be interpreted with caution. Several factors influence the data including deterioration of the receptor during tumor handling [11], differences in receptor level in different parts of the same tumor [11], differences in tumor cellularity and *in vivo* blockade of the receptor by circulating estradiol [12].

It has been suggested by Wittliff *et al.* [13] that part of these difficulties in quantification may be overcome if only the estradiol receptor sedimenting as an 8S complex in sucrose gradients is considered significant. Our data in Fig. 1 do in fact demonstrate that the 8S complex indicated with arrows was exclusively demonstrated in tumors with high specific binding capacity. However, several patients with gratifying remissions demonstrated only 4S receptor in their tumor. The presence of 8S receptor complex is an indirect measure of high specific binding capacity. This information may also be obtained with other methods such as electrofocusing and since we think that electrofocusing offers better quantification we have selected this method for receptor quantification in a prospective study in which the value of prophylactic tamoxifen therapy is explored in operable mammary carcinomas with defined receptor levels.

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Association of Carcinoembryonic Antigen (CEA) and Influenza Antigen

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Abstract—*An avian influenza A virus (FPV) was adapted to human bronchial carcinoma cells containing carcinoembryonic antigen (CEA) at their surface. Influenza hemagglutinin and CEA were physically associated at the level of the cell membrane. In the envelope of the mature FPV particle no substantial amount of CEA could be found. The important fraction of viral oncolysates for immunotherapeutic purposes—where an association of tumor associated and viral antigens is required—thus appears to be the cell membrane fraction. These experiments show furthermore that a cell surface constituent can be attached to erythrocytes by influenza virus hemagglutinin. This attachment by hemagglutinin might be an interesting tool for experimentation with other cell surface constituents.*

INTRODUCTION

AUGMENTATION of the immunogenicity of tumor cells by infection with viruses has been shown in several systems [1–4]. Enveloped viruses were used for this purpose, since the association of viral and tumor-associated antigens (TAA) at membrane sites is apparently important for such augmentation [5]. The experiments described in this report were undertaken to see whether this association occurs mainly at the cell membrane or in the viral envelope. An answer to this question is important in the preparation of viral oncolysates for immunotherapeutic trials such as the one being carried out by the Swiss group for clinical cancer research in acute myelogenous leukemia [6].

In this paper we demonstrate that a physical association of carcinoembryonic antigen (CEA) and influenza hemagglutinin (HA) takes place. This association is found mainly at the level of the cell membrane.

A human bronchial carcinoma cell line which produces CEA [7] was infected with avian influenza A virus. This system was chosen for several reasons:

1. Cells: these cells contain CEA at their surface (Ellison and Robertson, unpublished observation), the location where influenza viruses are budding. In addition, CEA is the most intensely studied human tumor-associated antigen and it can easily be measured [8, 9].
2. Virus: the hemagglutinating antigen of influenza A virus is a surface antigen and can readily be determined.
3. An avian influenza A virus (FPV) was chosen since its replicative ability in human tumor cells was known [10–14].

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MATERIAL AND METHODS

Cells, CEA determination, virus

The bronchial carcinoma cells (BEN) and the methods of CEA determination have been described previously [7,8]. The fowl plague virus (FPV; A/Turkey/England/63, Hav 1, Nav 3, Langham strain) which we used in these experiments had the following passage history: obtained from H. G. Pereira, World Influenza Centre, Mill Hill, London, England, after four allantoic passages; the virus was adapted to grow in human epithelial tumor cells, the last passage being in a primary culture of a human bronchial carcinoma [10].

FPV replication in BEN cell monolayer cultures

The replication of FPV in BEN cell monolayer cultures (as shown in Fig. 1) was carried out in the following way: A 25 cm^2 Falcon plastic flask (Falcon Plastics, Oxnard, Calif.) containing a monolayer of BEN cells was washed twice with 5 ml of Eagle's minimum essential medium (MEM). One third of a milliliter of a FPV suspension (for starting titer, see Fig. 1) was then added. The infected culture was incubated for 20 min at 36°C

Table 1. FPV infected and control BEN cell cultures. FPV infectivity, FPV hemagglutinin, and CEA in the 1000 g culture supernatant at different times of incubation

Hours of incubation		FPV infectivity*	FPV hemagglutinin†	CEA‡
4	FPV infected	1.5	0	0.141
	control	-	-	0.118
24	FPV infected	1.0	0	0.429
	control	-	-	0.430
48	FPV infected	6.7	5	0.440
	control	-	-	0.461
72	FPV infected	7.5	160	0.446
	control	-	-	0.451

*Log_{10} $TCID_{50}$ per ml.
†Hemagglutinating units per ml.
‡μg per ml.

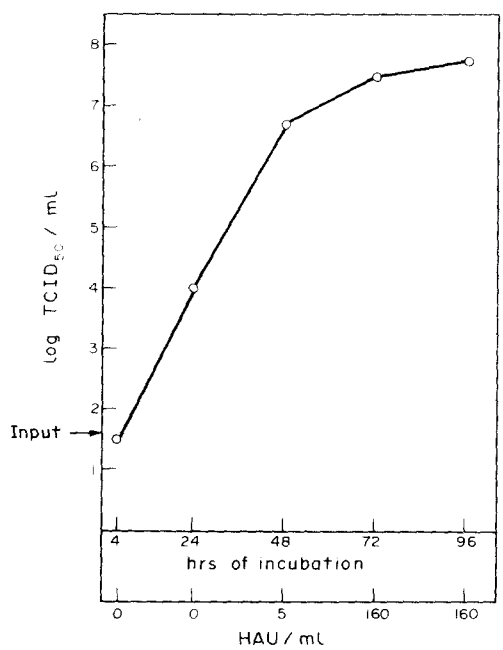

Fig. 1. FPV replication in a BEN cell monolayer culture. $TDID_{50}$: Dose with which 50% of tissue cultures are infected. HAU: Hemagglutinating unit.

with agitation every 5 min for virus adsorption. MEM, 4.67 ml, with 2% fetal calf serum was then added and incubation continued. The supernatant was removed (and replaced by 5 ml of fresh medium) at 4, 24, 48, 72 and 96 hr of incubation. The removed samples were put into sterile glass ampoules and stored at −80°C until infectivity titration could be performed.

Virus infectivity assays. Hemagglutinin determinations

Virus infectivity assays were carried out in Falcon 3040 microplates containing chicken embryo fibroblast monolayer cultures. The monolayers were infected with 0.1 ml of serial 10-fold dilutions of virus in MEM without serum. Six holes per dilution were used. After 5 days of incubation at 37°C the monolayers were checked for cytopathogenic effect. Holes with incomplete destruction of the monolayers were tested for hemagglutinin. The 50% tissue infectious dose ($TCID_{50}$) was calculated according to Reed and Muench [15]. The hemagglutinin titrations were effected using WHO standard procedures. Values are given in hemagglutinating units (HAU).

RESULTS

After two passages in BEN cells the infectivity titers of FPV in BEN cells reached almost 10^8 infectious particles per ml after 96 hr of incubation (Fig. 1) whereas the hemagglutinin titer reached 160 HAU/ML. FPV replication was as good as in chicken embryo fibroblast monolayer cultures [10]. The FPV therefore appeared to be optimally adapted to grow in BEN cells.

In a first experiment FPV infectivity, FPV HA, and CEA were determined in the 1000 g culture supernatant at different points in time. As seen in Table 1, CEA content did not differ in supernatants of FPV infected cultures from that in uninfected control cultures.

Since in this first experiment there was no obvious association of mature FPV (i.e. FPV which had left the cell) and CEA, centrifugation and hemadsorption experiments were undertaken to see whether there was an association at earlier stages of FPV development. By 2000 g centrifugation the bulk of cell bound viral antigen was separated from mature viruses. Table 2 shows that substantial amounts of FPV-HA (77%) and of CEA (58% in FPV infected cultures, 40% in control cultures) were sedimented at 2000 g. By further centrifugation of the 2000 g supernatant at 50,000 g (see Table 3) the infectivity and the hemagglutinin could again be concentrated (40 × and 100 × respectively). The CEA concentration on the other

Table 2. FPV infected and control BEN cell cultures (after incubation for 75 hr). FPV infectivity, FPV hemagglutinin, and CEA before and after low speed centrifugation (2000 g)

	$TCID_{50}$	HAU	CEA
Before centrifugation			
FPV infected culture	9.0	16,600	442
control culture	—	—	626
After centrifugation			
2000 *g* supernatant			
FPV infected culture	8.9	1020	186
control culture	—	—	340
2000 *g* sediment			
FPV infected culture	8.4	12,800	255
control culture	—	—	250
Percentage sedimented by 2000 *g*			
FPV infected culture	25%	77%	58%
control culture	—	—	40%
Times concentrated by 2000 *g*			
FPV infected culture	12 ×	40 ×	30 ×
control culture	—	—	21 ×

Four 75 cm^2 Falcon plastic flasks containing monolayers of BEN cells were infected with $10^{2.5}$ $TCID_{50}$ of FPV, whereas the monolayers of the other two flasks served as controls. Each flask contained 15 ml of MEM with 2% fetal calf serum. After 75 hr of incubation at 36°C the flasks were put at −20°C until the content was frozen. After thawing there were no cells attached to the plastic bottle any more. The content of the two virus infected flasks as well as the one of the two control flasks was pooled and centrifuged at 2000 *g* for 30 min at 4°C. Infectivity, hemagglutinin, and CEA were determined in the supernatants and the sediments. The numbers given in the Table are total amounts of $TCID_{50}$ (log_{10}), HAU and CEA (μg) per two 75 cm^2 Falcon flasks.

Table 3. FPV infected and control BEN cell cultures (75 hr of incubation). FPV infectivity, FPV hemagglutinin and CEA before and after high speed centrifugation (50,000 g)

	$TCID_{50}$	HAU	CEA
Before 50,000 *g* centrifugation (after 2000 *g*)			
FPV infected culture	8.9	660	186
control culture	—	—	340
After 50,000 *g* centrifugation			
50,000 *g* supernatant			
FPV infected culture	8.7	0	147
control culture	—	—	298
50,000 *g* sediment			
FPV infected culture	8.3	400	4
control culture	—	—	2
Percentage sedimented by 50,000 *g*			
FPV infected culture	25%	61%	2.2%
control culture	—	—	0.6%
Times concentrated by 50,000 *g*			
FPV infected culture	40 ×	100 ×	4 ×
control culture	—	—	1.04 ×

The 2000 *g* supernatants obtained as described in the legend to Table 2 were centrifuged at 50,000 *g* for 60 min at 4°C. Infectivity, hemagglutinin and CEA were determined in the supernatants and the sediments. The numbers given in the Table represent the total amounts of $TCID_{50}$ (log_{10}), HAU and CEA (μg) per two 75 cm^2 Falcon plastic flasks.

Table 4. FPV infected and control BEN cell cultures. Adsorption to and release from human erythrocytes of FPV hemagglutinin and CEA of the 2000 g sediment

		HAU	CEA
FPV infected			
2000 *g* pellet:	before adsorption to erythrocytes	20,480	39
	after adsorption to erythrocytes	1600	27
	adsorbed to erythrocytes	18,800	12
	released from erythrocytes (after 37°C)	12,640	4
Control			
2000 *g* pellet:	before adsorption to erythrocytes	—	39
	after adsorption to erythrocytes	—	41
	adsorbed to erythrocytes	—	0
	released from erythrocytes (after 37°C)	—	0

2000 *g* sediments were obtained as described in legend to Table 2. One milliliter of virus infected or control sediments were mixed with 9.0 ml MEM with 2% fetal calf serum containing 3.2×10^9 washed human 0 Rh negative erythrocytes. This mixture was kept at 0°C for 60 min with gentle agitation every 10 min. Thereafter the erythrocytes were spun down at 100 *g* for 15 min at 4°C. Nine milliliters of the supernatant were then carefully removed for HA and CEA assays. To the remaining 1.0 ml which contained the 3.2×10^9 erythrocytes 9.0 ml MEM with 2% fetal calf serum were added. These 10 ml were then incubated at 37°C for 60 min with gentle agitation in a water batch. Afterwards the erythrocytes were centrifuged again at 100 *g* for 15 min at 4°C. Released HA and CEA were determined in the supernatant.

The numbers given in the Table represent total amounts of HAU and CEA (μg).

hand did not increase very much in the 50,000 *g* sediment (4× for the FPV infected cultures, 1.4× for the control cultures).

These centrifugation experiments suggest a physical association of FPV–HA and CEA in the 2000 *g* sediment. If this were the case then CEA of this fraction of the FPV infected cultures and not of the control cultures should—by the action of the viral hemagglutinin—adsorb to erythrocytes and—through the viral neuraminidase—be released again from erythrocytes. Two thousand *g* sediments and 2000 *g* supernatants of FPV infected and control cultures were therefore mixed with human erythrocytes. The results of such an adsorption and release experiment with 2000 *g* sediments are shown in Table 4. From the pellet of FPV infected material 31% of the CEA was adsorbed to erythrocytes whereas from the control pellet none was adsorbed. After incubation of the erythrocytes at 37°C one third of the CEA was released again from the red cells which had been in contact with the FPV infected material. No CEA was released from erythrocytes which had been mixed with uninfected control material. In the 2000 *g* supernatants, no CEA adsorption to erythrocytes could be detected either with the FPV infected or with the control material.

DISCUSSION

Previous work from this laboratory showed adaptation of an avian myxovirus (FPV) to different human tumor cells [10–14]. In the present report we have demonstrated that this virus can also be adapted to grow in a human bronchial carcinoma cell line (BEN). Since these cells contain CEA at their surface we were in a position to examine if there was a physical association of CEA and viral antigen during the budding process of the virus. Low speed centrifugation of viral oncolysates (i.e. virus-destroyed tumor cells) where most of the cell membrane fragments sediment indicated an association of viral HA and CEA at the cell membrane (Table 2). This association was confirmed by hemadsorption (Table 4). Concentration of free FPV but almost no concentration of CEA by ultracentrifugation (Table 3) suggested a lack of CEA and HA association in the envelope of mature FPV. The slight concentration of CEA achieved by 50,000 *g* with the virus infected material (Table 3) might have been due to residual membrane fragments not sedimented at 2000 *g*. Our results accord with the results of immunization experiments where it was shown that membranes [16] but not purified viruses [17] from virus infected tumor cells protected animals against challenge with viable tumor cells.

In conclusion: fowl plague virus hemagglutinin and carcinoembryonic antigen (CEA) are physically associated at the level of the cell membrane. In the envelope of the mature FPV particle no substantial amounts of CEA were associated with viral HA. Immunoelectron

microscopic experiments which we are undertaking at present will show whether there is any CEA at all in the envelope of FPV grown in BEN cells.

These experiments may have the following consequences:

1. The important fraction of the viral oncolysate for immunotherapeutic purposes where an association of tumor-associated and viral antigens is required, appears to be the 2000 ***g*** sediment, which contains most of the cell membranes. Preparation of viral oncolysates on a large scale for clinical studies [6] could therefore be simplified by avoiding ultracentrifugation for collecting mature virions if other tumor-associated antigens behave similarly to CEA.
2. The association of CEA with influenza virus hemagglutinin allows the attachment of CEA to the surface of erythrocytes. Attachment of cell surface constituents to erythrocytes might be useful for the study of such constituents including the detection of immunological reactions against them.

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Sequential Studies of Melanoma Leukocyte-Dependent Antibody Activity in Melanoma Patients*

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Abstract—*Sequential assays of leukocyte dependent antibody (LDA) activity against melanoma cells were carried out on melanoma patients with localized tumour growth before and after surgical removal of their tumour. In approximately a third of the patients, LDA activity appeared in their sera after surgical removal of their tumour and this showed a gradual decline over several months. The absence of melanoma LDA in their sera prior to surgery appeared to result from the presence of serum blocking factors against LDA in their sera prior to surgical removal of the melanoma. Melanoma LDA in the pre-surgery sera, could be shown by removal of blocking factors by gel chromatography procedures and the "unblocked" LDA appeared specific for melanoma cells. In a second group of patients, no LDA activity was seen and a third group showed high levels which were present before surgery and showed no significant decline after surgical removal of their melanoma. The second group of patients were mainly males who also had a high incidence of regional lymph node involvement whereas the third group were all multiparous women. The present data do not allow evaluation of the prognostic significance of these respective patterns of LDA activity.*

The results indicate the melanoma LDA can be detected in a high proportion of patients at an early stage of tumour growth. They confirm our previous findings of blocking factors against melanoma LDA in patients with disseminated melanoma and establish that the LDA blocking activity has biological significance in relation to tumour growth in melanoma patients.

INTRODUCTION

ANTIBODY dependent cell mediated cytotoxicity (ADCC) induced by leukocyte dependent antibody (LDA) has now been described against a number of tumours in experimental animals [1–6] and against several tumours in human subjects, such as bladder carcinoma [7], leukaemia cells [8] and melanoma cells [9–11]. We have recently shown that LDA against melanoma cells can be shown in the sera of melanoma patients and that several different antigens can be defined on melanoma cells by these assays which show limited cross-reactivity between melanoma cells from different individuals [11]. It was also found that in patients with disseminated melanoma, LDA activity was often completely blocked by factors in their sera and that the frequency of LDA in melanoma patients may therefore be much higher than that revealed by assay of their unfractionated sera [12, 13].

In the present study, the incidence of melanoma LDA in patients with early melanoma was examined and sequential studies were carried out before and after surgical removal of melanoma to detect any changes in this antibody activity related to the change in disease activity induced by surgery. Our results showed that several patterns of LDA activity were observed in melanoma patients. One pattern appeared related to the disappearance of blocking activity against LDA in the sera of melanoma patients after surgery and changes in LDA activity induced by these factors appeared to correlate with clinically determined melanoma growth in these patients.

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MATERIAL AND METHODS

1. *Patients*

The study included 13 female patients and 20 male patients who presented to the Melanoma Unit for surgical removal of primary melanoma (Stage 1) or melanoma localised to regional lymph nodes (Stage 2). Pertinent clinical data on these subjects is shown in Table 1. Control sera were obtained from four normal laboratory workers and serial samples were taken from eight patients with malignancies other than melanoma who were admitted for surgical removal of their tumour.

2. ^{51}Cr-*release assays*

The assay procedures used have been described in detail in previous reports [11, 13] and are described briefly below.

(i) *Target cells* (*TCs*). Melanoma cells from the continuous human line MM 200 were used as the main target cell in these studies. This cell line, as well as other melanoma lines used in the study, were kindly supplied initially by Dr. J. Pope of the Queensland Institute of Medical Research. These cell lines, including the melanoma antigens expressed on their surface, have been described previously [11]. Autologous studies were carried out on melanoma cultures established from fresh surgical specimens, as described previously. The non-melanoma tumour cultures were also established from fresh surgical specimens and

Table 1. Clinical data on patients in LDA study

Patients	Age	Sex	Pregnancies	Clinical stage	Site of primary	Duration to recurrence (months)	Present status	Trans-fusions	Blood group
Group A									
JM	47	M	—	2	T	(11) —	0	2	A+
DT	23	M	—	2	L	—	0	—	O+
SD	54	M	—	2	T	11	0	—	A+
RGJ	42	M	—	2	H	(38) 1	3	2	O−
GD	79	M	—	1	H	—	0	—	O+
WF	74	M	—	1	T	—	0	—	
AB	49	M	—	1	T	—	0	—	O+
PMc	50	F	3	1	T	—	0	—	
MC	47	F	1	2	L	5	D	—	A+
EM	56	F	3	1	L	—	0	1	O+
JS	29	F	6	2	L	(26) 6	3	2	O+
SUD	22	F	3	1	H	—	0	—	
Group B									
HP	72	M	—	2	T	7	0	2	O+
CH	72	M	—	2	A	—	0	—	A−
CC	54	M	—	2	H	4	0	—	A+
GA	54	M	—	2	L	(120) —	0	—	O−
DJ	24	M	—	2	H	(7) 1	D	—	O+
CS	25	M	—	2	H	(8) 6	D	2	O+
KJ	55	M	—	2	T	(5) —	0	2	O+
GM	37	M	—	1	L	—	0	2	A+
OD	67	M	—	2	L	(14) —	0	2	B+
NB	58	M	—	2	T	5	2	—	A+
GH	58	M	—	1	T	—	0	—	O+
AC	21	M	—	1	H	—	0	—	O+
SP	67	M	—	2	T	—	0	—	O+
JS	31	F	1	2	T	—	0	—	B+
PS	45	F	4	2	A	—	0	2	A+
LH	60	F	0	1	A	—	0	—	A+
Group C									
FG	39	F	2	1	L	—	0	—	
LF	38	F	4	1	T	—	0	1	O+
AH	61	F	6	1	L	(24) 5	2	—	A−
FH	44	F	7	1	L	—	0	—	O−
GD	50	F	3	2	L	(10) —	0	—	O+

T = trunk; H = head and neck; L = leg; A = arm; stage 1 = primary melanoma; stage 2 = primary melanoma + regional lymph node involvement; stage 3 = disseminated melanoma; D = dead.
*Figures in parentheses indicate duration to first recurrence when surgery was for second recurrence.

were treated as described for the melanoma cultures. All cultures were grown in RPMI 1640 (Gibco) supplemented with 20% foetal bovine serum (FBS) (Australian laboratory services batch 42) and were harvested by incubation with 0.25% Trypsin for 15 min immediately prior to use.

(ii) *Effector cells.* Mononuclear cells were obtained from defibrinated blood samples by centrifugation on Hypaque–Ficoll mixtures of specific gravity 1.078 after methods described by Boyum [14]. All sera were tested with effector cells from both the patient and normal laboratory personnel. The cells were re-suspended in RPMI 1640 + 10% FBS at a concentration of 10^6/ml for use in the assays.

(iii) ^{51}Cr *labelling.* TCs were labelled with ^{51}Cr by incubation with 100 μCi of Na_2CrO_4 (Amersham, Bucks, UK) for 2 hr. The cells were washed twice in 25 ml of Eagles minimal essential medium (MEM) (CSL Melb.) and resuspended in RPMI + 10% at a concentration of 10^4/ml for use in the assays.

(iv) *LDA assays.* Sera were assayed for LDA by addition of 100 μl at the dilutions indicated to cultures containing 5×10^3 TCs in 500 μl. This was followed by the addition of 5×10^5 effector cells in 500 μl. The cultures were in duplicate 12 × 75 mm round bottomed plastic tubes (Sterilin, Filtrona, Melbourne), which were capped and incubated overnight for 16 hr at 37°C in 7% CO_2 in air. Assays were terminated by centrifugation for 7 min at 400 ***g*** and 500 μl of the supernatant were removed for estimation of ^{51}Cr release. Samples were counted in an automatic Wallac 1280 gamma counter.

The percentage of the ^{51}Cr release was estimated by the formula

$$\frac{a \times 2.2}{a+b} \times 100$$

where $a = {}^{51}$Cr counts in the supernatant background counts and b = counts in tube with remaining supernatant and cells-background counts. Results are expressed as percent ^{51}Cr release above or below the baseline ^{51}Cr release from the TCs in the presence of the effector cells alone. (This level of cell mediated cytotoxicity (CMC) varied from subject to subject and has been discussed previously [15].

3. *Gel exclusion chromatography*

Whole sera were separated into several fractions on the basis of molecular size using Sepharose CL-6B (Pharmacia) in a column 2.6 × 70 cm, (type k26/70), (Pharmacia). Two milliliters of whole serum was applied to the column and eluted with 0.5 ml saline in Sorensen's glycine buffer at pH 2.8 containing 0.02% Sodium Azide by upward flow at a rate of 0.5 ml/min at 4°C. Fifty fractions of 6 ml each were collected.

Ultraviolet (u.v.) absorbance of the fractions was measured at 280 nm on a Unicam SP1800 u.v. Spectrophotometer and the fractions were pooled into 4 groups according to molecular size, on the basis of chromatogram results. The large molecular weight (mol. wt) fractions were concentrated back to 2 ml in an Amicon Diaflo cell, using a PM10 membrane and the small mol. wt fraction using a UM-2 membrane. They were then dialysed twice against 500 ml 0.9% NaCl. Immunoglobulins in each were identified by immunodiffusion on Tri-Partigen Plates (Hoechst).

Statistical analysis

Student's *t*-test was used to compare the percent of ^{51}Cr release of cultures with and without LDA activity and findings were considered significant when $P < 0.05$.

RESULTS

1. *Patterns of melanoma LDA activity in sera from melanoma patients*

In Fig. 1 the effect of adding serum from subject DT taken before and 10 days after surgery, to cultures of the MM 200 TC and effector cells from DT is shown. Before surgery serum at a 1/10 dilution had slight blocking activity of the direct CMC. After

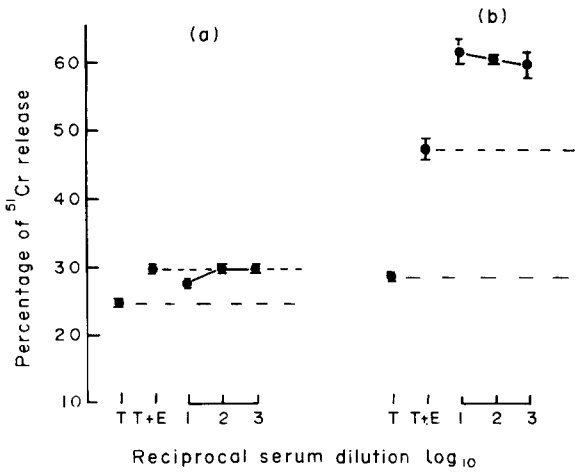

Fig. 1. Serum LDA activity and serum blocking of spontaneous CMC of subject DT (a) before and (b) 10 days after surgery. T = spontaneous release from MM 200 TCs alone. T+E = ^{51}Cr release from TC due to spontaneous release and direct CMC of effector cells. Values above this line indicate LDA activity in the serum and values below this line indicate blocking of the spontaneous CMC. Values are means ± 1 standard error (S.E.).

surgery the serum had LDA activity to a titre exceeding $1/10^3$. The direct CMC of DT was also increased after surgery. The appearance of melanoma LDA activity after surgery was seen in 12 of the 33 patients in the study and the results at a single dilution of serum at various time periods after surgery, is shown for 10 of these patients in Fig. 2. (Each serum was tested at three dilutions, as in Fig. 1, but for clarity only the activity at the 1/10 serum dilution is shown in Fig. 2 and 3). Sera from these patients taken prior to surgery had no LDA activity and sometimes had blocking of the spontaneous CMC of the effector cells. By 2–4 weeks after surgery, LDA activity was detectable in their sera and this activity showed a gradual reduction over the next few months. Clinical data on the 12 patients in this group is shown in Table 1. One female patient in this group died approximately 5 months after the initial operation and three other subjects have had evidence of regional lymph node recurrence (a, b, c and d correspond to patients MC, DT, SD and EM in Table 1, respectively).

The LDA activity in sera taken before and after surgery from the other patients is shown in Fig. 3. Sixteen patients had no LDA activity in their sera or had blocking of the spontaneous CMC to melanoma cells prior to surgery. After surgery, reduction in blocking activity against spontaneous CMC occurred in several patients but no LDA activity was seen. Clinical data on the 16 patients in this group in Table 1 indicates that there was a predominance of males in this group and most patients had regional lymph node involvement. Two of the patients died during the period of study and there were three other patients with regional lymph node recurrences (a′, b′, c′ and d′ correspond to patients GM, HP, GA and LH in Table 1, respectively).

Also shown in Fig. 3 are the results of studies on 5 patients in which melanoma LDA activity was present before surgery and and persisted essentially unchanged during the period of study. In Table 1 it is seen that all 5 subjects were women and all were multiparous. One of the 5 has had local recurrence of melanoma. (Constant levels of LDA activity against the MM 200 target cell were also seen in 13 other women who have attended the Clinic over a 2-yr period and who did not have pre-surgery serum samples available for LDA assays. All but one of these women were multiparous. Two of these patients have since died from disseminated melanoma. One patient has had liver metastases for approximately 15 months and remains in reasonable health on no therapy. Another patient had apparent complete remission from disseminated melanoma concurrent with the administration of methyl-CCNU.)

2. *LDA activity in the 7s IgG containing fractions of melanoma sera*

To further understand the reason for absence of melanoma LDA activity in the pre-surgery sera, the IgG fraction of the sera were tested in parallel with the unfractionated sera against

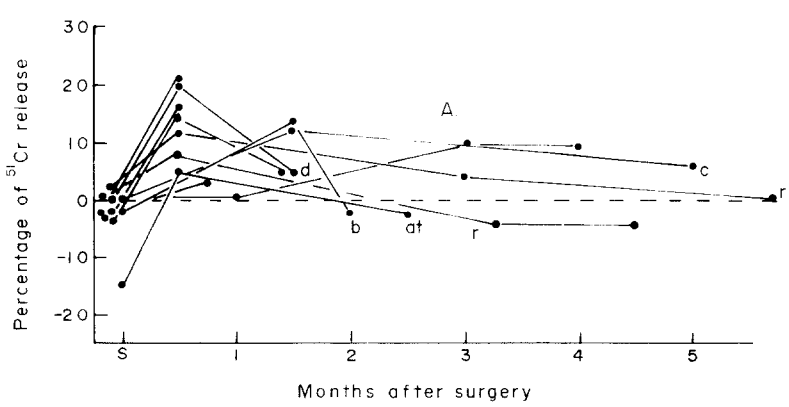

Fig. 2. Serum LDA and blocking of spontaneous CMC against the MM 200 target cell before and after surgery. The spontaneous CMC in the absence of serum is taken as the baseline and values above this line indicate LDA activity and values below the line, blocking of CMC. The spontaneous ^{51}Cr release from the TC in the presence of the effector cells ranged from 30 to 55%. The effects of the sera on CMC in the Fig. are those recorded at a dilution of 1 in 10. †=patient who subsequently died of recurrent melanoma. r=patient with recurrence of melanoma. (S.E. of the points $< \pm 2\%$, $P < 0.05$ for points showing ^{51}Cr release of 5% above or below base line in absence of serum).

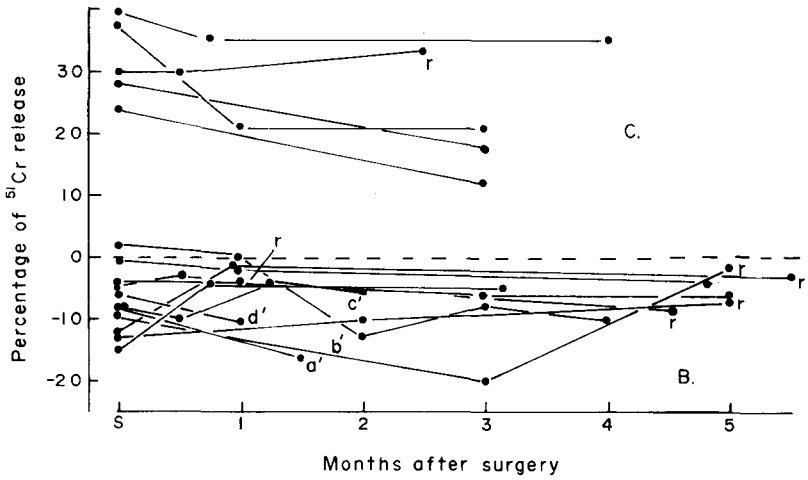

Fig. 3. Serum LDA and serum blocking activity against spontaneous CMC of melanoma patients before and after surgery. (See also legend to Fig. 1). In pattern B no serum LDA was seen and varying levels of blocking of CMC are indicated by depression of CMC below the level in the absence of serum. In pattern C relatively constant levels of LDA are seen before and after surgery. r=Patients who had recurrence of melanoma. (S.E. of points $< \pm 2\%$. $P<0.05$ *for points showing* ^{51}Cr *release of* 5% *above or below base line in absence of serum).*

the MM 200 target cell. One such study is shown in Fig. 4 where it can be seen that the IgG fraction at the time of surgery had strong LDA activity even though the unfractionated sera had no LDA activity. At 3 weeks after surgery, the LDA activity of the IgG fraction was less and weak activity was also seen in the unfractionated sera. By 2 months, no LDA activity was seen in either the IgG fraction or the whole sera.

In Table 2, the results of similar melanoma LDA assays on fractionated and unfractionated sera before and after surgery for 4 patients from group A and 4 from group B are shown. (The patients selected for study are indicated a, b, c and d in Fig. 2 and a', b', c' and d' in Fig. 3. The assays were carried out at 3 dilutions of the IgG fractions but only the 1/10 dilution is shown for clarity and because these results accurately reflected the general trend of the results at the other dilutions.) All patients showing LDA activity after surgery in group A had LDA activity in the IgG fraction prior to surgery and this showed a gradual reduction after surgery. Patients in group B however, did not have LDA activity detected in the IgG fraction of sera taken prior to or after surgery.

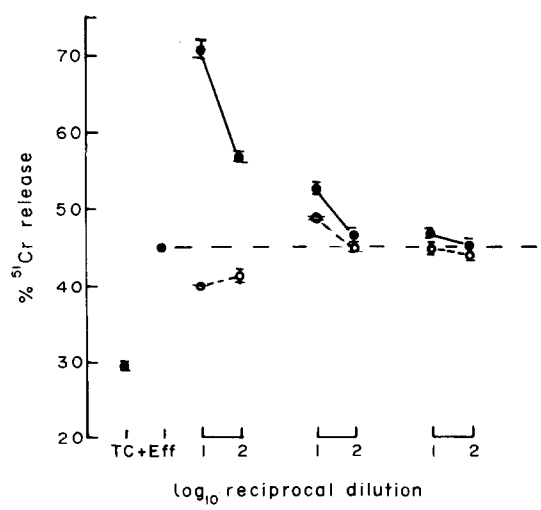

Fig. 4. LDA activity of unfractionated sera (indicated by dashed lines) compared to the IgG fraction separated from sera at the time of surgery, 3 weeks and 7 weeks after surgery.

Table 2. Melanoma LDA activity of the IgG fraction of melanoma sera before and after surgery

Melanoma patients	Days after surgery				
	0	14	28	42	60–90
Group A					
(a) MC	25±2	7±1	—	—	2±2
(b) DT	12±1	12±0.5	—	8±0.5	—
(c) SD	9±3	12±1	—	12±2	8±1
(d) EM	10±2	12±1	—	10±1	2±5
Group B					
(a') GM	0±1	2±1	—	0	—
(b') HP	−2±5	0±1	—	—	−5±2
(c') GA	2±5	3±2	—	5±1	—
(d') LH	2±2	—	2±1	—	0±2

Values indicated are the percent ^{51}Cr release above or below the CMC values alone at a dilution of 1 in 10±1 S.E.

3. *LDA activity in sera from control subjects*

Sera were taken from two groups of control patients at the same time intervals before and after surgery as the melanoma patients discussed above. One group were melanoma patients who had previously had their melanoma removed 6–8 weeks before admission for a second operation for wider excision and skin graft of the site of the original primary. As shown in Table 3, no LDA activity appeared in the sera of these subjects after surgery, and no LDA activity was seen in the 7s fraction from 2 of these subjects.

The second group of controls were patients with malignancies of various types, as indicated in Table 2. The patients with Hodgkin's disease and acute myeloid leukaemia underwent surgery for splenectomy. The other subjects had curative removal of primary tumours. The whole sera of many of these subjects had blocking activity of spontaneous CMC to the MM 200 target cell but no detectable LDA activity. The 7s fractions of pre-surgery and post-surgery sera from the patients with carcinoma of the breast and larynx also did not show LDA activity against melanoma cells, indicating that the absence of LDA activity was not due to blocking factors in their sera.

4. *LDA activity of melanoma sera against non-melanoma cells*

The specificity of LDA revealed in the melanoma sera after surgical removal of the melanoma was examined by assay of 7 of the most reactive sera from group A and a 7s fraction from 1 patient in group B (GA) against several different types of carcinoma cells and cells from the Chang liver cell line. All sera were tested at 3 dilutions from 1/10 to 1/1000. The results at the 1/10 serum dilution are shown in Table 4 and indicate very little LDA activity against these non-melanoma cells. Two positive reactions were seen with serum GD against an ovarian and a lung carcinoma and one positive reaction by GA against the squamous cell carcinoma (SCC) cells. Insufficient amounts of the sera from other patients in group A were available for extensive specificity assays but all were negative against the Chang cell and 1 breast carcinoma cell. The sera from patients in group C did not react with the Chang cell, 1 breast carcinoma or 1 ovarian carcinoma.

5. *LDA activity of unfractionated and the 7s fraction of melanoma sera against different melanoma cells*

To examine the possibility that the absence of LDA activity in patients in group B may be due to the absence of cross-reactivity with the MM 200 TCs, the 7s fraction and the unfractionated sera of 8 subjects from group B were tested in LDA assays against other melanoma cell lines and when possible, against

Table 3. LDA activity of sera from control subjects against melanoma cells

Subject		CMC in absence of serum†	Percentage ^{51}Cr release above or below baseline CMC ± 1 S.E.					
			Pre-surgery		2–4 weeks post-surgery		8–12 weeks post-surgery	
			WS	IgG	WS	IgG	WS	IgG
KS	WEG	10 ± 0.5	0	0	0		−15 ± 1.5	
GS*	WEG	8	−2 ± 1	−3 ± 0.5	0		−3 ± 1.5	
CL*	WEG	12	2 ± 0.5	−3	−11		−11 ± 2	
SJ	WEG	5 ± 0.5	−5 ± 1.5		−1			
NN	WEG	20 ± 2	−7		−6		0	
DE	*Ca.* Breast	6 ± 1.5	−10 ± 1.5	−4 ± 2	−10 ± 1		−9	−3
JD	*Ca.* Breast	16 ± 1.5	−8	−2 ± 1	−10	−4 ± 0.5		
RR	*Ca.* Larynx	14 ± 2	−8	−3 ± 1	−6 ± 1	0		
WP	SCC	15 ± 1	−14 ± 1	0 ± 1	−7 ± 0.5			
KG	AML	15 ± 2	−12 ± 2		−12 ± 1			
GH	Hodgkin's	15 ± 2	−6 ± 1	0 ± 1	−6 ± 0.5			
PL	SCC	22 ± 3	−12 ± 1.5	−2 ± 1	−15 ± 1.5			
JC	SCC	16 ± 1	−8 ± 0.5	1 ± 2	−5 ± 1			

*Effector cells from normal controls. WS = unfractionated serum.
†CMC = % ^{51}Cr release above that from MM 200 TC alone. Spontaneous release from target cell ranged from 20 to 35%.
WEG = wide excision of biopsy site and graft.
SCC = squamous cell carcinoma.

Table 4. LDA activity of melanoma antisera against non-melanoma cells

Subject	Percentage of ^{51}Cr release above or below baseline CMC (±1 S.E.)							
	Ovarian carcinoma (37±2.5) (6)	Ovarian carcinoma (13±1.5) (2)	Breast carcinoma (36±3.0) (12)	Breast carcinoma (23±0.5) (2)	S.C.C. (41±2.5) (4)	Chang cell (26±1) (6)	Insulinoma (15±2.5) (2)	Lung (30±2.0) (1)
EM	0±1	2±0.5	—	0	0	−4±1	3±0.5	0±1.5
DT	−3	0	0±1.5	0±0.5	0	0	0	0±1.8
JC	0±1	2±2	—	0±0.5	0±2	−3±0.5	−2	3±2
JM	2±0.5	—	0±0	—	0±0	0	—	—
GD	12±5	3±0.5	0±1	0±0.5	−4±0.5	0	4±0.5	7±0.5
GA(IgG)	−4±2	—	−6±1.5	—	7±1	—	—	0±1.8
SD	—	—	−5±0	0±2.5		−2±1	4±1	0±2
SUD	0	0	—	0±1.5	—	−1±1	0	0±2

Figures in the Table indicate LDA or blocking of spontaneous CMC at a serum dilution of one tenth. The figures in parentheses indicate the ^{51}Cr release from the TCs. The numbers in parentheses below these indicate the CMC values above the spontaneous release from the TCs. SCC = squamous cell carcinoma.

the autologous target cell. The results showed that this possibility was unlikely in that a low level of LDA activity (<5% above base line) was seen only against two autologous TCs.

6. *LDA activity in patients with recurrent melanoma*

During the period of study, several patients followed since surgery had recurrence of their melanoma. The LDA activity in their sera, prior to detection and after surgical treatment of the recurrent melanoma, is shown in Fig. 5. Three patients (JM, SD and JR) who had previously shown melanoma LDA after surgery, had a gradual reduction in LDA activity to baseline levels or below the baseline levels at the time of detection of the recurrent melanoma. After surgical removal of the recurrence, LDA activity appeared again in the sera of JM and JR but not SD.

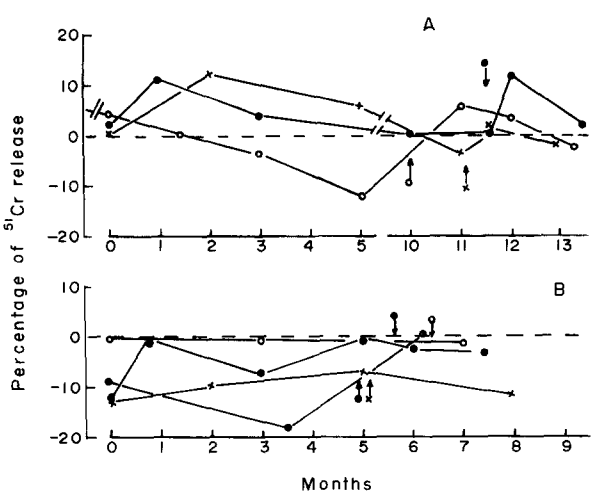

Fig. 5. Serum LDA activity against the MM 200 melanoma cell after surgery for recurrent melanoma. The arrows indicate time of surgery for second recurrence. Serum dilutions 1 in 10. (S.E. of points < ±2%. P<0.05 for points showing ^{51}Cr release of 5% above or below base line in absence of serum).

Patients who had previously had pattern B (HP, CC, CS and KJ, Table 1) had no consistent change in the LDA activity prior to detection of the recurrent melanoma. After surgical removal of their tumours, the pattern of LDA activity continued to show either blocking of spontaneous CMC or had no effect on the level of CMC. No LDA activity to the MM 200 line was seen after surgery in either the 7s fraction of the unfractionated sera of these patients.

DISCUSSION

It is apparent from the above study that LDA activity can be detected after surgical removal of melanoma in approximately a third of melanoma patients and that failure to detect this activity at the time of surgery in these patients was due to the presence of blocking factors against LDA in their sera. On theoretical grounds, absence of LDA prior to surgery could result from other factors, such as sequestration of antibody by the growing tumour mass or result from failure of antibody production before surgical removal of the tumour. However, the demonstration that melanoma LDA activity can be shown in the IgG fraction of the sera before surgery is against these possibilities and suggests that failure to detect melanoma LDA at this time is due to the presence of factors in the unfractionated sera which block the LDA activity as described in our previous reports [12, 13]. Similar blocking of ADCC in an experimental animal tumour model has also recently been described [16]. The nature of these factors is currently under investigation but our pre-

liminary results have shown that they are glycoproteins of 15,000 mol. wt and these factors can also be found in the supernatants of melanoma cell cultures. This evidence therefore, suggests that they are surface antigens released from the melanoma cell.

Transfusions were given at the time of surgery in several of the patients but this could not account for the rise in LDA in most of the patients. There was also no significant difference in the number of people receiving transfusions in either of the three groups of patients. Variation in the activity of antibody dependent (AD) effector cells also did not account for the increased LDA activity. Most patients in the study had no significant increase in AD effector cell activity after surgery when tested in parallel studies against sensitized Chang cells and there were no significant differences in AD effector cell activity between the three groups of subjects (Manuscript in preparation).

The LDA activity revealed in the 7s fraction of sera taken prior to surgery, or in the sera of melanoma patients following surgery appeared relatively specific for melanoma. Sera from patients with tumours unrelated to melanoma did not show LDA activity to melanoma cells, before or after surgery and did not have LDA activity in the 7s fraction of their sera. Conversely, when the sera or the 7s fraction of melanoma sera were tested against non-melanoma tumour cells and the Chang cells there was no, or very little, LDA activity against these cells. Many of the sera with melanoma LDA activity were also from young or middle aged men so that prior sensitization to HLA or foetal antigens during pregnancy was not possible and in most cases there was no previous history of blood transfusions. The decrease in the antibody titres after surgical removal of the melanoma also suggested that the continued presence of antigens on the melanoma cells were required for antibody production.

Despite the detection of these disease-related changes in LDA assays of sera from some melanoma patients, it is apparent that this activity was not reflected in the LDA assays of sera of many of the patients in the study. In some patients this appeared to be largely due to an absence of detectable LDA against the melanoma target cell. Early recurrence of melanoma was noted in 5 of the 16 patients in this group, which suggests that in some the absence of detectable LDA may have been due to continued presence of occult melanoma. If so, the antibody may have been sequestered by the tumour or the method of "unblocking" LDA used in this study may not have been adequate to remove the blocking activity from the LDA in these patients. It was also noted that there was a high incidence of regional lymph node involvement in this particular group of subjects and perhaps this may have resulted in suppressor activity against antibody production.

In previous studies it was noted that melanoma antigens appeared to vary between different melanoma cells and that not all melanoma sera reacted with the melanoma TC used in this study. Many of the unreactive sera were therefore tested against other melanoma cells but no significant increase in LDA activity was seen against the other melanoma cells. These results therefore suggest that melanoma LDA was absent in the sera from these patients and that failure to detect LDA was not merely due to selection of target cells without cross reacting melanoma antigens.

The patients showing a high level of melanoma LDA in group C were also of interest in that they were all multiparous women. It was shown previously that sera taken from women during pregnancy had antibodies to melanoma cells which could be absorbed on foetal tissue and that some of the reactivity of sera from women with melanoma could also be absorbed on foetal tissue. Extensive absorption studies of this nature have not been repeated on all the sera in the present study but 2 of the 5 sera had reduced LDA reactivity after absorption on foetal tissue. These results suggest that the high, relatively constant, LDA activity in these women may have resulted from prior sensitization to foetal antigens during pregnancy. We have previously reported that prior sensitization during pregnancy to foetal antigens may account for the better survival of women who have had previous pregnancies compared to those who have not [17]. The high levels of LDA however, did not confer absolute protection against recurrence from melanoma in the present patients in that one of the five women in this study and 3 of 13 women from among the general clinic patients with similar high levels of LDA, developed recurrent melanoma despite high LDA levels.

The present data do not allow any significant comparison to be made of the relative survival rate between the patients in this study, although there is a suggestion that patients with the high constant levels of melanoma LDA may have a better survival than patients with no detectable LDA activity. Further studies of these patients are continuing in order to answer this question.

Although the biological significance of melanoma LDA in protection against melanoma is not clear at this stage, the present study has

shown that the assays can be used to monitor the presence of blocking activity in the sera of melanoma patients. This activity (which appears to be melanoma antigen) has shown good correlation with disease activity in the present study and in studies in progress in which blocking activity has been tested against melanoma sera known to be reactive in LDA assays. The use of these assays may therefore provide a much needed monitor of disease activity in these patients which can be used to detect early recurrence of melanoma and assist in the planning of more effective therapeutic regimes against the tumour.

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Adriamycin and Adriamycin–DNA in Inoperable Bronchogenic Carcinoma. A Randomized Study with Cyclophosphamide Vinblastine

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Abstract—*In a randomized study on 59 patients with inoperable bronchogenic carcinoma, the chemotherapeutic efficacy and toxicity of ADM–DNA was compared with that of free ADM and that of the association cyclophosphamide–vinblastine.*

No significant differences in initial chemotherapeutic response could be observed between the groups of patients treated respectively with cyclophosphamide–vinblastine, free adriamycin or adriamycin–DNA. The cardiotoxicity of ADM seems however reduced by its combination with DNA, since no signs of cardiotoxicity were observed, even in the 15 patients who received a total dosage of adriamycin–DNA exceeding 500 mg/m².

INTRODUCTION

THE PRINCIPLE of lysosomotropic chemotherapy of cancer has been described and tested experimentally by one of us with daunorubicin–DNA [1] and adriamycin–DNA [2].

These drugs are presently undergoing clinical trials in the treatment of leukemia, non-Hodgkin lymphomas [3] and breast cancer [4]. The present study attempts to assess the value of this lysosomotropic chemotherapy in the treatment of inoperable bronchogenic carcinoma for which efficacy and toxicity of adriamycin (ADM) and adriamycin–DNA (ADM–DNA) are compared with those of a classical drug association cyclophosphamide–vinblastine (CPM–VBL).

MATERIAL AND METHODS

A total of 78 cases of inoperable bronchogenic carcinoma have been introduced in the study.

In every patient, the diagnosis was proved by histological and/or cytological methods and any possibility of surgical treatment had been excluded.

We also excluded the cases previously treated by surgery radio or chemotherapy.

All patients hospitalized in the pneumologic unit of the University of Louvain in Godinne between 10/1/1974 and 6/1/1976 and responding to these criteria have been included in the study.

From the 78 initial patients, only 59 have been retained for the appraisal of results since they had been clinically and radiologically checked after the 2nd drug perfusion.

The 19 patients lost correspond with 3 deaths and 16 refusals of treatment before the 2nd perfusion.

The patients were randomized in two groups: Group I (29 cases): every 3 weeks, i.v. perfusion, lasting 1 hr, of cyclophosphamide (CPM) (900 mg/m²) with an i.v. bolus of vinblastine (VBL) (9 mg/m²). Group II (30 cases): every 3 weeks, i.v. perfusion of ADM (free or combined with DNA) (75 mg/m²). Group II patients were randomized again in two subgroups: II_A (16 patients) ADM–DNA, prepared as described earlier [1] from the beginning of the treatment; II_B (14 patients) free ADM for the 3 first perfusions (at a 3 weeks interval) and from the 4th perfusion on ADM–DNA.

These two subgroups II were constituted in order

(1) to compare the chemotherapeutic efficacy of free and complexed ADM after three perfusions;

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(2) to evaluate the cardiotoxicity of ADM–DNA;
(3) to compare the efficacy of ADM–DNA alone or preceded by free ADM with that of a standard CPM–VBL combination.

In another trial, still in progress, the preliminary results of which will be reported here, cases of undifferentiated carcinoma were randomized in 2 therapeutic groups, analogous to the two subgroups II described above, including ADM–DNA in the first subgroup and free ADM followed by ADM–DNA in the order.

During the evolution of the disease and according to the toxicity phenomena the interval between the perfusions was progressively increased from 3 to 4, 6 and 8 weeks. The total number of perfusions varied between 2 and 14, with a median value of 6.5.

The therapeutic response was evaluated according to the criteria of Kenis [5]. In this evaluation, the regression of the tumor was only taken into consideration, with exclusion of the atelectasic retractions or the resorption of infectious processes. The tumor size was monitored by X-rays and each X-ray plate was examined separately by 2 chest physicians.

In case of discordant interpretation, the opinion of a third observer was considered as determinant.

The following types of therapeutic response were considered:

(1) Complete remission (CR): complete disappearance of the tumor, for at least 1 month.
(2) Partial remission (PR): decrease of the tumor greater than 50% of the product of the two diameters, without progression of any other lesion and without appearance of any new lesion during a period of at least one month.

A partial remission is evenly stated when a marked or unequivocal decrease (3/4 or more) is observed in non-measurable lesions.

(3) Non total failure (NTF): decrease of the tumor, as in partial remission, but lasting less than 1 month or definite decrease in some lesions, occurring together with progression of other.
(4) Total failure (TF): either decrease of the tumor inferior to 50%, or status quo or progression of the disease.

Groups 1 and 2 are considered together as remissions, while groups 3 and 4 are classified as "failures".

The toxicity of therapeutic regimens was assessed with great care in the 4 following areas:

(1) Alopecia
(2) Gastrointestinal disturbances: these were considered as minor when they involved anorexia, nausea or infrequent vomiting; and qualified as major if nausea and vomiting occurred for one week or more.
(3) Bone marrow depression: drug perfusion was postponed or the doses were decreased when the number of neutrophilic polymorphonuclear leucocytes was inferior to 1800 per μl and/or when the platelets count was lower than 100,000 per μl.
(4) Cardiotoxicity: electrocardiograms and dosage of creatine phosphokinase (CPK) were performed systematically, before every perfusion of ADM or ADM–DNA.

As summarized in Table 1, the groups

Table 1. Characteristics of patients

	Group I	Group II		
	Total group I CPM–VBL	Total group II	Subgroup A ADM + ADM–DNA	Subgroup B ADM–DNA
Number	29	30	14	16
Age—limits	41–77	44–77	48–77	44–73
—mean	60	64	64	64
—median	61	65	64	67
Histological type				
squamous cell	24	15	8	7
anaplastic (small cell)	4	11	5	6
undifferentiated (large cell)	1	3	1	2
glandular		1	—	1
Tumor localization				
central	21	19	9	10
peripheral	8	11	5	6

and subgroups were very similar with regard to number of patients, age, tumor localization (central or peripheral).

The number of undifferentiated small cell epithelioma is however higher in group II than in group I.

Data on duration of survival were evaluated by life table analysis [6]. Chi-squared analyses were performed for comparison between the results of both groups.

RESULTS

1. *Tumoral response to chemotherapy*

Table 2 summaries the objective responses observed. The percentage of remissions (23.3%) due to ADM and/or ADM–DNA is not significantly superior to that obtained with the CPM–VBL association (17.2%) ($P=0.20$). The remission rate observed in subgroup II_B (free ADM followed by ADM–DNA) seems higher than that of subgroup II_A (ADN–DNA alone); this difference is however not significant ($P=0.71$).

Various results are obtained in relation to the histological types of the tumor (Table 3).

Well differentiated tumors (squamous cells carcinoma and adenocarcinoma) respond better to CPM–VBL association (21%) than to ADM/ADM–DNA (6%). On the contrary, undifferentiated (anaplastic small and large cell

Table 2. Objective response to treatment

	Group I	Group II		
	Total group I CPM–VBL	Total group II	Subgroup A ADM + ADM–DNA	Subgroup B ADM–DNA
Complete remission	—	1	1	—
Partial remission	5	6	4	2
Non total failure	2	—	—	—
Total failure	22	23	9	14
Remissions	5/29	7/30	5/14	2/16
	17.2%	23.3%	35%	12.5%
	$P=0.20$ $\chi^2=0.06$		$P=0.71$ $\chi^2=1.14$	

Table 3. Objective response to treatment related to histological type

			Well differentiated tumors	
	Group I	Group II		
	Total group I CPM–VBL	Total group II	Subgroup A ADM + ADM–DNA	Subgroup B ADM–DNA
Remissions	5	1	1	—
Failures	19	15	7	8
Remission rate	5/24	1/16	1/8	0/8
	21%	6%		
	$P=0.58$ $\chi^2=0.66$			
			Poorly differentiated tumors	
Remissions	—	6	4	2
Failures	5	8	2	6
Remission rate	0/5	6/14	4/6	2/8
	0%	43%		
	$P=0.77$ $\chi^2=1.46$			

carcinomas) are more sensitive to ADM/ADM–DNA (43%) than to CPM–VBL. As shown in Fig. 1, the actuarial survival curve of patients is similar in groups I and II ($P=0.279$). There is however a significant difference ($P<0.01$) between the survival curves of patients with response to treatment and those without response (Fig. 2).

2. *Toxicity*

As summarized in Table 4, alopecia is almost always present and complete in patients treated with ADM/ADM–DNA. With CPM–VBL, alopecia is less frequent (62%) and less complete. Gastrointestinal troubles are equally frequent in both groups as well as bone marrow depression, which is remarkably mild.

No cases of cardiotoxicity are observed, neither in patients treated with ADM–DNA nor in those receiving first ADM alone. The total dose of drug varies between 150 and 1.050 mg/m^2 (median 480); 15 patients receive a total amount equal or superior to 500 mg/m^2.

Table 4. *Toxicity*

	Group I	Group II
Alopecia	18 (62%)	27 (90%)
Gastrointestinal complaints		
Minor	13	17
Major	5	3
Total	18 (62%)	20 (66%)
Bone marrow depression	5 (17%)	8 (26%)

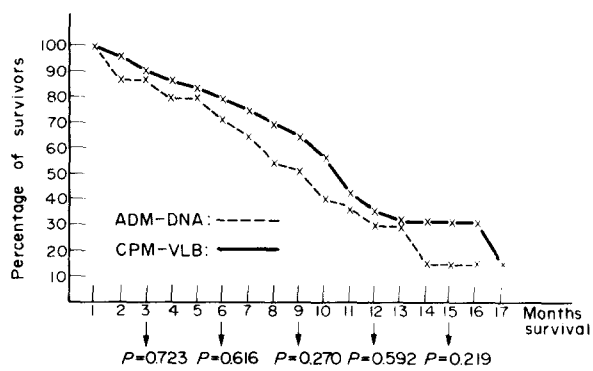

Fig. 1. *Survival curves of patients treated with ADM–DNA and with CPM–VLB. No statistically significant difference.*

DISCUSSION

Our results show that in inoperable bronchogenic carcinoma ADM–DNA (preceded or not by ADM) induces a therapeutic response comparable to that of a combination of CPM–VBL.

Results from the literature [7–10], summarized in Table 5, show that the rate of remission obtained by us with ADM–DNA preceded or not by free ADM (23%) falls in the range of remissions obtained by other authors with free ADM.

From data of Table 2, it seems that some difference exists between the response rate in patients treated with free ADM followed by ADM–DNA and that recorded in patients receiving only ADM–DNA.

These differences are however not significant. Preliminary results from another study still in progress on a greater number of patients with undifferentiated carcinoma (Table 6) show however that after three perfusions remission rates with ADM–DNA look more similar to those obtained with free ADM ($P=0.57$).

This very important aspect of the ADM–DNA combination is presently submitted to further studies since one of the possible drawbacks of the macromolecular-linked-drug could diffuse more slowly in badly irrigated solid tumors and could have more difficulties in crossing capillary barriers than the free drugs.

Moreover, our knowledge of the endocytic properties of lung tumor cells is still partial.

Table 6. *Study still in progress. Preliminary results after* 3 *perfusions*

Anaplastic small cell bronchogenic carcinoma		
	Group I ADM + DNA	Group II free ADM
No. of cases	12	13
No. of remissions	7	10
No. of failures	5	3
Remission rate	58%	77%

$\chi^2=0.32$; $P=0.57$.

Table 5. *Remission rate (complete and partial) obtained with adriamycin in inoperable bronchogenic carcinoma*

		No. of remissions / No. of patients	% of remissions
Bonadonna	1971	4/14	28.6%
Blum and Carter	1974	44/229	19%
Cortes *et al.*	1975	9/31	29%
Kenis	1975	16/50	32%
Selawry	1975	68/377	18%

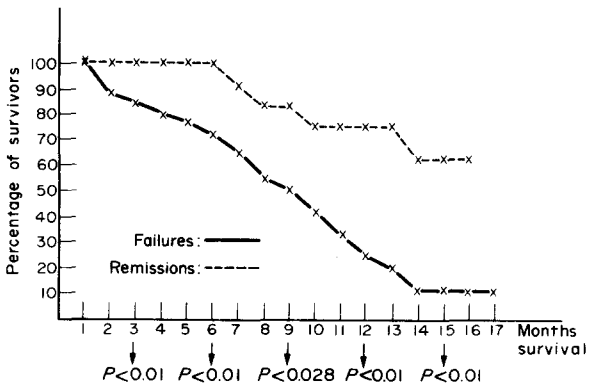

Fig. 2. Survival curves comparing patients in whom remission was induced and those in whom it was not. Survival in the latter group was significantly shorter.

The first results of the study in progress indicate that the trend in favor of free ADM is less marked than in the present study: linking ADM to DNA seems not to reduce markedly the chemotherapeutic efficacy of ADM in bronchogenic carcinoma.

In the present study, the results obtained with ADM are much better in undifferentiated epitheliomas (43% remissions) than in squamous cell carcinomas (6% remissions). This difference is just statistically significant ($P=0.05$). Our observations contrast with those obtained on a greater number of patients by Blum and Carter [7] indicating a greater sensitivity of the squamous cell carcinomas to ADM and with those of Selawry [10] suggesting an equal sensitivity of both histologic types to ADM.

The use of ADM linked to DNA does not reduce the frequency and extent of alopecia. Stomatitis, a frequent complication of adriamycin treatment [7], was not observed in our material, nor were more infrequent complications like cutaneous rashes [11] and fibrinolysis [12].

Although systematical E.C.G. controls and CPK assays were performed, patients of group II did not show any cardiotoxicity: these results gain still more significance if one considers that, as shown in Table 7, 15 patients received more than 500 mg of ADM/m^2: the cardiotoxicity of free ADM increases indeed very much when the administered amount exceeds 500 mg/m^2, as shown from the data of Gilladoga *et al.* [13] summarized in Table 7 and from the data of Lefrak *et al.* [14] indicating that when the total dosage of ADM exceeds 500 mg/m^2 cardiotoxicity appears in about 20% of the cases and even in 30% when the total amount exceeds 550 mg/m^2.

In our results, one can find a first indication that linking ADM to DNA leads to a decrease of cardiotoxicity of ADM in human patients.

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Table 7. Cardiotoxicity of adriamycin

Adriamycin mg/m^2	Gilladoga *et al.* (1976) ADM			Present series ADM-DNA/ADM		
	No. of patients	No. of cardiotoxicity	%	No. of patients	No. of cardiotoxicity	%
501–600	21	0	0	4	0	0
601–800	11	1	9	5	0	0
801–1000	7	2	29	5	0	0
>1000	3	1	33	1	0	0
>800	10	3	30	6	0	0

$\chi^2=0.68$; $P=0.40$.

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Bladder Carcinogenesis Using Bilharzia-Infested Swiss Albino Mice

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Abstract—*Experimental studies were carried out on female Swiss albino mice to evaluate the possible role of: liver injury, urine inborn carcinogens and local irritation of bladder mucosa in the development of bilharzial bladder cancer. In mice, the liver and intestine are only involved by parasite, but the urinary bladder is spared. Mice with induced liver injury due to either: bilharzial infestation alone, hepatocarcinogens alone (2-naphthylamine (2-NA) or 2-acetylaminofluorene (2-AAF)), or both together, did not develop bladder tumors. Also, mice receiving 1% indole (a tryptophan precursor) either alone, or after bilharzial infestation, did not show any change in bladder mucosa. Conversely, the insertion of glass beads in the bladder induced carcinomas in 26.6% of mice after 70 weeks. The induction of bladder tumors by glass beads was enhanced by 2-AAF treatment (40%), but not by bilharzia infestation (25%), 2-NA (27%) or indole (27%). The urinary bladders with glass beads were invariably the seat of bacterial infection (E-coli and gram + ve cocci) and showed epithelial hyperplasia and metaplasia.*

INTRODUCTION

THE ETIOLOGIC relationship between chronic bilharzial cystitis and bladder cancer has been established. Since the original report of Ferguson [1], many valuable contributions have been added [2–5], but the exact mechanism of carcinogenesis is still unknown.

Different theories have been postulated including the mechanical effect of ova [1], miracidial toxins [2], associated chronic inflammation [3] and the presence of certain urinary carcinogenic agents such as tryptophan metabolites [4–7].

The aim of the present work is to evaluate the role of liver injury and mechanical irritation of bladder mucosa in the production of bladder cancer in an experimental animal model. Liver injury was induced either by bilharzial infestation and/or chemical carcinogens.

MATERIALS AND METHODS

Animal model and experimental groups

Mice were selected for this study, as in such animals, we get hepatic bilharzial infestation without bladder involvement, since the blood vessels of the mouse bladder are too small to allow the parasite to reach the bladder. The possible role of mechanical irritation of the bladder by the passage of ova was imitated by surgical insertion of glass beads in the bladder cavity.

Female Swiss albino mice, 6–8 weeks old, about 20 g bred at Cairo Cancer Institute were used. The mice were randomly divided among the different groups (Table 1). Animals were maintained on a standard diet containing 20% protein and were given water *ad libitum*. In groups given oral chemical carcinogens, 1% 2-naphthylamine (2-NA) and 0.06% 2-acetylaminofluorene (2-AAF) were mixed with the diet separately. Indole, a tryptophan precursor, was mixed with the diet in a concentration of 1%.

The majority of mice were sacrificed after 70 weeks. However, in the groups receiving chemical carcinogens (2-NA and 2-AAF), the experiments were terminated after 30 weeks due to the development of severe toxicity. The experiment was started by 480 mice. A total of 130 animals were either sacrificed or found dead during the experiment, thus leaving 350 mice at the termination of study.

Bilharzial infestation

The mice were immersed for 1 hr in water containing cercaria of *S. mansoni*, 20–30 per ml. Six weeks later, the stools of mice were

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Table 1. *Frequency of bladder carcinomas in Swiss albino mice*

Group	Period treatment (WK)	Number of mice	Tumors Number	Tumors Percent
i. Normal control	70	45	0	0
ii. Liver injury:				
(a) Bilharzia	70	46	0	0
(b) 2-Naphthylamine (2-NA)	30	20	0	0
(c) 2-Acetylaminofluorene (2-AAF)	30	20	0	0
(d) Bilharzia + 2-NA	30	17	0	0
(e) Bilharzia + 2-AAF	30	22	0	0
III. Local bladder irritation: (glass beads)	70	30	8	26.6
IV. Bladder beads + liver injury:				
(a) Beads + bilharzia	70	20	5	25.0
(b) Beads + 2-NA	30	18	5	27.0
(c) Beads + 2-AAF	30	25	10	40.0
V. Tryptophan precursor:				
(a) Indole	70	25	0	0
(b) Indole + bilharzia	70	20	0	0
(c) Indole + beads	70	22	6	27.7
(d) Indole + beads + bilharzia	70	20	5	25.0

examined for the presence of ova to confirm the infestation. Only mice with positive ova in the stool were selected for the experiments.

Insertion of glass bead in the bladder

Under intraperitoneal nembutal anaesthesia [8], a spherical glass bead with rough surface, about 7–9 mg, was inserted into the lumen of the bladder following the technique of Allen *et al.* [9].

Pathologic techniques

At autopsy, the left lobe of liver and urinary bladder were excised, then fixed overnight in 10% neutral formalin in saline. The fixed bladder was transected horizontally at 3 different planes. Tissues were processed by routine paraffin embedding and hematoxylin and eosin staining.

RESULTS

Pathology of liver

All the mice infested with *Schistosoma* showed bilharzial granulomata in the portal areas, but the normal liver cytology and lobular pattern were preserved. Around the bilharzia ova, histiocytes, fibroblasts lymphocytes and eosinophils were arranged in a concentric pattern. A black granular bilharzial pigment was also observed in the granulomatous areas and bilharzia worms were at times seen in portal veins. Bile ducts showed moderate to marked hyperplasia. The liver of mice treated with 2-naphthylamine (2-NA) or 2-acetylaminofluorene (2-AAF) showed mild fatty degeneration and cellular pleomorphism with hyperchromatic nuclei. However, no hepatomas were observed.

Pathology of urinary bladder

In the normal bladder, the transitional epithelium was 2–4 layers in thickness, with a surface layer of large cells. No epithelial changes were observed in mice infested with bilharzia or fed 2-NA or indole. A mild patchy epithelial hyperplasia was encountered in mice fed 2-AAF. Conversely, the bladders with glass beads showed marked transitional cell hyperplasia, both of surface epithelium and cell nests of Brunn. In addition, squamous metaplasia and columnar metaplasia were commonly observed. An inflammatory reaction was always present in the subepithelial stroma (lamina propria). Bacteriologic studies on urine revealed evidence of *E-coli* and gram positive *cocci*.

Malignant tumors of the urinary bladder only developed in the groups with glass beads (Table 1). Enhancement of tumor development occurred in mice fed 2-AAF, but not with other treatments (2-NA, indole or bilharzial infestation). All the tumors were deeply infiltrative. Of the 39 induced tumors, 21 were transitional carcinomas, 8 were transitional in type with focal squamous differentiation, and 10 were anaplastic with areas of spindle cells or sarcomatoid pattern. In spite of the advanced stage, and high grade of the majority of tumors, no distant metastases were observed.

DISCUSSION

Impairment of liver function in bilharzial patients, with subsequent disturbance of tryptophan metabolism, was considered a possible factor in bladder carcinogenesis [4]. However, hepatic bilharziasis affects mainly mesenchyme and spares liver cells, hence, very little change in liver function is observed except in very advanced cases [10–12]. In the present study, liver injury due to either: bilharzial infestation, hepatocarcinogens, or both together, failed to induce bladder tumors or atypical urothelial changes. Feeding indole (tryptophan precursor), also failed to produce changes in bladder mucosa.

In the present report, bladder tumors only developed in the groups of mice with intravesical glass beads. The induction of carcinomas was enhanced by treatment with 2-acetylaminofluorene, but not enhanced by bilharzial infestation, 2-naphthylamine or indole. These findings suggest that local factors are more important than systemic factors for bladder carcinogenesis in the present model.

The various hyperplastic, metaplastic and neoplastic changes observed in the bladders with glass beads are similar to those previously reported by Roe [13]. A comparable spectrum of lesions was also reported in the urinary bladder of bilharzial patients [14]. However, in human, squamous metaplasia and squamous carcinoma that follow bilharzial cystitis tended to show more abundant keratinization than those observed in mice.

Two factors are possibly operable in bladder carcinogenesis following glass bead implantation, namely: (a) chronic mechnical irritation and/or (b) the associated bacterial infection. The bacterial types identified (*E. coli* and *cocci*) were known to be capable of synthesizing nitrosamines from nitrate and secondary amines [15, 16]. These nitrosamines proved to be potent carcinogens in different laboratory animals [17].

Nitrate is a normal dietary component, being present in large amount especially in agricultural areas in Egypt, where nitrate is commonly used as a fertilizer. Secondary amines are also produced by intestinal flora and excreted in urine. Therefore, the environmental conditions of the Egyptian farmers, who are heavily infested with bilharziasis, associated with bacterial cystitis and drinking water with high nitrate content, should raise a great possibility of *in situ* formation of different carcinogenic nitrosamines in the bladder.

At present, further studies are carried out at the Cairo Cancer Institute to evaluate the possible factors involved in the induction of bladder cancer in bilharzial patients.

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Repair of Potentially Lethal Radiation Damage in Mouse Embryo

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Abstract—*The ability of mouse embryo cells to repair potentially lethal damage was studied. Female mice of the* 15th *gestation day were irradiated with X-rays or neutrons* (30 MeV *deuteron→berylium*) *and embryos were removed at various times thereafter. Surviving fractions of embryo cells determined by colony formation method* in vitro. *Survivals increased rapidly if embryos were left* in utero *for more than* 2 hr *after X-irradiation and reached a plateau at* 4 hr, *indicating that embryo cells can repair potentially lethal damage. The magnitude of this kind of repair was found dose-dependent.*

INTRODUCTION

REPAIR of potentially lethal radiation damage was first demonstrated by Phillips and Tolmach in exponentially growing cultured cells [1]. This kind of repair has been observed in stationary phase cells and in animal tumors [2–4]. Potentially lethal damage refers to the radiation-induced damage which is originally lethal but can be repaired in a certain post-irradiation condition or by some kinds of post-irradiation treatments. Several factors which may affect the repair of potentially lethal damage have been extensively studied [5–7]. Post-irradiation conditions which are suboptimal for cell growth are reported to favor this kind of repair. Conditions which are formed by hypoxic foci in tumor tissue may facilitate this kind of repair. Accordingly, it was predicted that only hypoxic tumor cells can repair potentially lethal damage. As already reported, we tested it in an animal tumor system and found that not only hypoxic tumor cells but also aerobic tumor cells might be capable of repairing potentially lethal damage [8]. Our study also suggested that this kind of repair is, at least partly, attributed to the "cell-to-cell contact" *in vivo*. Therefore, further studies were made on the capability of normal tissue to repair the potentially lethal damage.

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MATERIAL AND METHODS

Mouse embryos were used throughout. Parent animals were 8–10-week-old C3Hf/He mice which were derived from our specific pathogen free (SPF) colonies. They were kept in an animal facility where SPF condition was maintained as well as constant temperature and humidity. Animals were provided sterilized purina pellets and chlorinated water *ad libitum*. Two days were allowed for mating and pregnant female mice were used for studies at the 15th gestation day.

An X-ray machine was operated at 200 kVp and 20 mA with a filter of 0.5 mm Cu and 1 mm Al. Target skin distance was 50 cm. Half value layer was 1.2 mm Cu. The dose rate measured by Victoreen thimble ionization chamber, was approximately 100 rad/min at the center of the animal. Neutrons were produced by bombarding 30 MeV deuterons to a thick berylium target. Animals were irradiated at TSD of 150 cm and the dose rate was $\simeq$ 50 rad/min. During irradiations, animals were kept in a lucite phantom which can hold 12 animals separately and whole bodies were irradiated without anesthesia.

Animals were sacrificed by cervical dislocation and embryos were removed. Single cell suspensions were prepared as follows: to begin with, head, extremities and internal organs were excised from each embryo and the rest of the body was minced by scissors.

The mince was trypsinized at 37°C for 45 min in a culture bottle which had 0.25% trypsin (Difco 1:300) in Ca and Mg-free Dulbecco's solution. The supernatant was removed and centrifuged at 1500 rev/min for 5 min. The sediment was resuspended with sufficient amount of complete F10 medium supplemented with 10% calf serum and antibiotics. Number of cells was counted on a hemocytometer and single cells were seeded in plastic petri dishes (Falcon 60 × 15 mm) after appropriate dilution of the cell suspension. Lethally irradiated embryo cells were used as a feeder layer: $5–8 \times 10^4$ embryo cells/dish were grown overnight and irradiated with 4000 rad immediately before plating of the test cells. They were incubated for 12–14 days in a CO_2 chamber at 37°C to which humidified 5% CO_2-air was constantly supplied. Colonies containing more than 50 healthy cells were counted as survivors after methylene-blue staining. The plating efficiency was usually ≃5%.

RESULTS

Female mice of the 15th gestation day received a whole body irradiation of 1000 rad. Embryos were excised at various times thereafter and cell survival was determined *in vitro*. As presented in Fig. 1, surviving fraction was progressively increased if embryos were allowed to remain *in utero* and reached a plateau at 4–6 hr, the value at 24 hr not indicating any significant change. It was noteworthy that survival of the embryo left *in utero* for 4 hr and that of embryo left

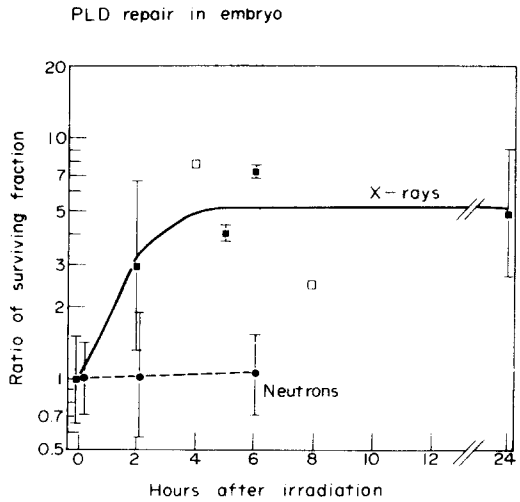

Fig. 1. The increase of survival of embryo cells as a function of time left in utero *after irradiation. Squares and circles indicate survivals after X-rays and neutrons respectively. Solid symbols are sums of more than 2 experiments while open squares indicate single experiment results. Vertical bars represent standard deviations.*

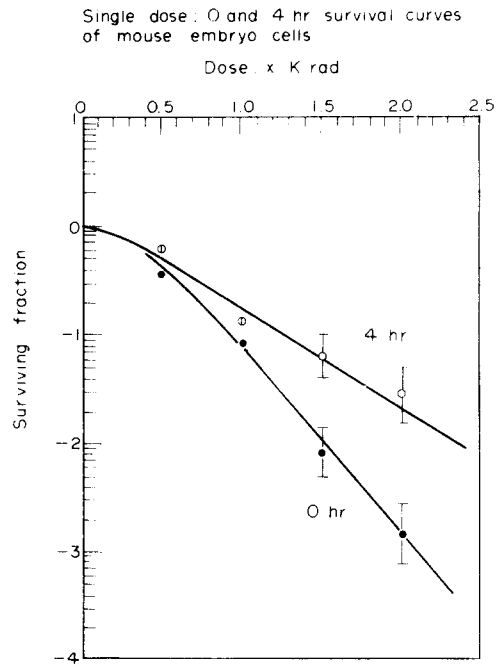

Fig. 2. Dose response curves of embryo cells irradiated with 200 kV p *X-rays and excised immediately (solid circles) or at* 4 hr *thereafter (open circles).*

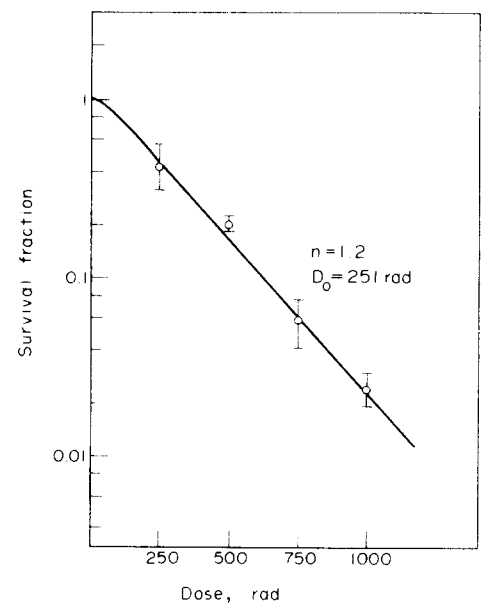

Fig. 3. X-ray dose-cell survival relation of embryo cells which were derived from primary culture of 15*-day-old embryos. Vertical bars indicate standard deviation.*

for 24 hr were consistent within an experimental error.

Dotted line in the same figure represents the survivals of embryo cells irradiated with neutrons of 500 rad and excised at various times thereafter. No increase of cell survival was observed, while a single neutron dose of 500 rad reduced survivals more effectively than an X-ray dose of 1000 rad.

Dose–response relations were assayed after single X-ray doses. Embryos were excised immediately or 4 hr after irradiation. Dose–cell survival curve of embryos left *in utero* for 4 hr after X-rays was found less steep than that of embryos excised immediately after X-rays. The D_0 value of the latter was 260 rad, while that of the former was 465 rad.

Our embryo cells appeared to be more radioresistant than other mammalian cells. Thus, the dose–cell survival relation of embryo cells which were derived from the primary culture of 15 day-old embryos and had been harvested *in vitro*, was examined. Single cells were plated and irradiated with X-rays 24 hr later. Number of colonies was counted at 14 days thereafter. As presented in Fig. 3, D_0 was 250 rad and was roughly comparable to that of embryos excised immediately after X-rays (Fig. 2).

DISCUSSION

Factors which facilitate the repair of potentially lethal radiation damage have been extensively studied *in vitro* and *in vivo* [5–7]. Among them, those applicable to normal tissue cells are: (a) the presence of non-proliferating or slowly proliferating cells and (b) the cell-to-cell contact, as suggested in our previous results.

Demonstration of this kind of repair *in vivo* requires comparison of surviving fractions of cells removed immediately after irradiation with that of cells left *in situ* for a certain time. Accordingly, only such cells which produce colonies *in vitro* can be tested for the repair capability and none of the normal tissue cells are susceptible to this test except for embryo cells. A disadvantage to use embryo cells for this kind of experiment was very low plating efficiency. However, this might be inevitable because many organs are already formed at the 15th gestation day and many cells are well differentiated and losing proliferative capability.

Our results clearly indicated that embryo cells can repair potentially lethal radiation damage over 4–5 hr after X-rays and the capability of repair was dose-dependent. It was also noteworthy that embryo cells were unable to repair the potentially lethal damage after neutron irradiation. All the results were consistent qualitatively with those obtained with animal tumors [3, 4, 7, 8] indicating that repair of potentially lethal damage is not a phenomenon which distinguishes normal tissues and malignant tissues.

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Dynamics of Immunity in Syngeneic Lymphoma-Bearing Mice

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Abstract—*Following a short interval of increased reactivity against third party antigens (allogeneic skin graft and sheep red blood cells) C3Hf/Bu mice bearing syngeneic lymphoma develop progressive immunodepression. In order to overcome this depression, the animals were injected with* 10^8 *lymph node or spleen cells from normal syngeneic donors. Lymph node cells restored skin allograft rejection time, as well as the number of plaque forming cells (PFC) in the spleen of tumorous host, but spleen cells failed to do so. In order to investigate the role of the tumor environment on the reactivity to third party antigens,* 10^7 *spleen cells from mice, at different stages of tumor disease, were injected into syngeneic, lethally irradiated recipients. The PFC values were higher in mice injected with spleen cells from tumor-bearing mice than in those injected with spleen cells from normal syngeneic donors, regardless of the stage of tumor growth. The results were discussed as to suggest that central defect in the depression of humoral and cell-mediated immunity in tumor-bearing host is due to altered T-cell population.*

INTRODUCTION

GENERALIZED depression of cellular and humoral immunity is associated with growth of syngeneic tumor and becomes stronger in advanced stage of tumor disease [1, 2]. Since the immunodeprived host is not able to reject allogeneic tumor, the probability of rejecting syngeneic tumor, which is much less antigenic, would be rather unlikely [3]. Furthermore, infection of mice with murine leukemia virus results in a marked impairment of immune responsiveness to various antigens–sheep red blood cells (SRBC), allografts, bacterial extracts [4, 5]. Besides tumor viruses, which could be considered to cause immunosuppression directly, various virus-associated factors, such as soluble extracts of tissues of virus-infected mice, or ascitic fluid, also resulted in immunosuppression [5, 6].

Current questions are: A. whether this immunodepression is caused by the tumor itself, B. if so, whether it is first localized in the microenvironment of the tumor, where interactions with host's immunocytes first occur and C. whether or not the immunodepression caused by the tumor itself provides it with a mechanism of evading the host immune surveillance [3].

To this end, changes of immunity against third-party antigens (allogeneic skin graft and SRBC) were investigated in C3Hf/Bu mice transplanted with syngeneic lymphoma cells, during tumor disease. The intention was to establish: (a) the degree of the immunodepression at different times of tumor disease, (b) the ability of different types of lymphoid cells to restore the host's immune reaction against the antigens and (c) the influence of the environment dominated by the tumor on the ability of immunocytes to react.

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MATERIALS AND METHODS

Experimental animals

Male mice of C3Hf/Bu and C57BL strains, bred in our colony were used. They were about 12–16 weeks old at the beginning of the experimental procedures.

Tumor

The tumor used in these experiments was a lymphoma which arose spontaneously in the thymus of a male C3Hf/Bu mouse [7]. The fourteenth isotransplant generation of a tumor transplant was kept in liquid nitrogen at −193°C. In the present experiment, "source" mice had been injected with frozen cells thawed immediately before implantation; cell suspensions were made from growing tumors of source animals and injected into the experimental mice. Following the injection of 10^6 viable tumor cells, the standard dose used,

the tumor became palpable 10–12 days later. Progressive growth continued and the host died about 40 days after the tumor became palpable. Morphological and immunological aspects of this lymphoma were previously described [8, 9]. Briefly, in comparison to the controls, the patterns of moderate immunological activity, comprising an increasing number of germinal centers as well as of lymphoblasts in the paracortical area, occurred in regional lymph node 2–3 days following lymphoma transplantation. This was followed by a decrease resulting in lymphoid tissue atrophy of the outer cortex in the third week of the disease. Simultaneously, the metastases of tumor cells occurred in the medulla and in the paracortical area and lymphoma cells destroyed the normal structure of lymph node about 10 days later. This chain of events occurred in other lymph nodes too, but it was postponed for about a week. Three days after tumor cell transplantation the number of lymphoblasts increased around the central arterioles in the white pulp of the spleen and increased number of germinal centers and plasma cells could be seen in hyperplastic white pulp 3–4 days later. This pattern of strong immunological activity persisted until the end of the third week following tumor transplantation, when the number of lymphoid cells started to decrease mainly around the central arteriole. Towards the end of the first month after tumor transplantation, the first tumor cells could be seen around the central arteriole and the tumor started to invade the organ, but more slowly than the lymph nodes.

Tumor cell suspension

Grossly intact pieces of the tumor isotransplant, removed from source mice, were minced, the mash was poured into a beaker containing cold Hanks' solution and mixed with a 10 ml syringe. The beaker was placed on ice so as to allow large particles to settle down. Five minutes later the supernatant was removed using a syringe with 22-gauge needle and passed through a Swinney filter into test tubes. The specimen was spun down on 150 ***g*** during 10 min, the supernatant was discarded and the pellet resuspended. All visible clumps were removed using a Pasteur pipette so that the cells were dispersed as a single cell suspension. The viability was tested by Trypan blue exclusion; more than 90% of tumor cells were scored as viable. A one tenth of a milliliter inoculum, containing 10^6 viable tumor cells, was injected subcutaneously into the right thigh of recipient mice.

Preparation of spleen and lymph node cell suspensions

Donor mice were killed by cervical dislocation and spleen, as well as lymph nodes (axillary, inguinal brachial and cervical) were harvested. The organs were minced in cold Hanks' solution using scissors. The mash was passed through the nylon patch into a beaker and placed on ice. Five minutes later the supernatant was discarded with a syringe, the pellet was passed in the test tube and washed 3 times on 150 ***g*** for 10 min each in Hanks' solution. The number of viable cells was determined and finally adjusted to 10^7 or 10^8 Trypan blue negative cells/ml.

Plaque forming cells assay

SRBC (Institute of Immunology, Zagreb) had been washed 3 times on 180 ***g*** for 5 min each and 10% suspension was made using Hanks' solution. A half milliliter of the suspension had been injected intraperitoneally to recipient mice. The immunologic response to SRBC was determined by counting the cells forming haemolitic antibodies (plaque forming cells—PFC) in the spleen according to the method of Jerne *et al.* [10] modified for use on microscopic slides [6]. SRBC had always been injected 4 days before the animals were sacrificed to determine PFC numbers. Lymphoma cells by themselves do not form plaques with SRBC (unpublished).

Skin graft technique

A piece of skin approximately 1 × 1 cm was applied to a suitably prepared graft bed and secured with a bandage [11]. The bandages were removed 7 days later and graft inspected daily. Graft was scored as rejected when there was no visible remaining epithelium.

Statistics

Mean values and standard errors were calculated for each group of experimental and control animals. The significance between two mean values was calculated by using the *t*-test.

RESULTS

1. *Dynamics of immunosuppression in lymphoma-bearing mice*

1.1. *Skin allograft rejection time.* C3Hf/Bu mice were grafted with skin from C57BL mice at various terms after tumor cell transplantation. On each particular day 10–12 tumorous mice and 4–5 normal mice were grafted. Results in all normal mice were pooled. The allograft rejection times are presented in Table 1. Mice grafted at the same time when tumor

cells were injected, or 2 days later, rejected the grafts like control mice and mice grafted 5 days after tumor transplantation rejected the graft even 1 day earlier than the controls without the tumor. However, from 9th day following the tumor transplantation, skin allograft survived up to 9 days longer on lymphoma-bearing mice. The bigger the tumor, the longer was the allograft survival.

1.2. *The number of PFC in the spleen.* One, 3, 9, 12, 15, 23, 30 and 40 days following lymphoma cell injection 4–6 mice from experimental group and 4–5 normal mice were sacrificed and PFC assay performed. All the mice have been injected with SRBC 4 days before. The PFC counts in experimental mice were then correlated with counts in the controls (at each particular day) and presented as a percentage of control values in Fig. 1. As presented, the spleens of C3Hf/Bu mice, injected 3 or 9 days before PFC assay with lymphoma cells, contained higher numbers of PFC to SRBC than control mice. However, towards the end of the second week following tumor cell injection, when the tumor became palpable, a pronounced decrease of the PFC

Table 1. *Survival of C57BL skin graft on lymphoma-bearing mice and normal C3Hf/Bu mice*

Days after injection of 10^6 tumor cells	Days of allograft survival (Mean ± S.E.)	Number of mice	Significance to controls (*t*-test)
Non-injected mice	11.4 ± 0.1	30	
0	11.9 ± 0.7	10	N.S.*
2	11.0 ± 0.5	11	N.S.
5	10.2 ± 0.4	11	$P<0.02$
9	12.9 ± 0.5	12	$P<0.01$
14	14.4 ± 0.4	10	$P<0.01$
21	15.4 ± 0.4	11	$P<0.01$
28	20.3 ± 0.6	11	$P<0.01$

*Not significant.

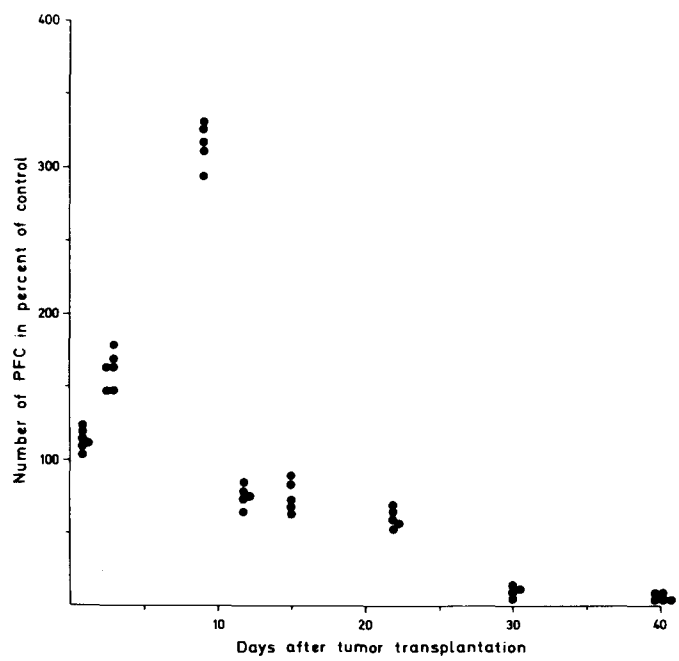

Fig. 1. *Number of PFC, expressed in percent of control, in C3Hf/Bu mice at particular days following lymphoma cell transplantation. Except on the* day 1, *the values are significantly different from the controls with* $P<0.05$, *on the* days 3, 12, 15 *and* 23, *as well as with* $P<0.01$ *on the* days 9, 30 *and* 40 *respectively.*

number in the spleens was noticed. About a month following tumor cell injection, the values reached very low levels, under 10% of the number in the spleen of normal animals.

Thus, initial stimulation of the reaction to third party antigens (skin allograft and SRBC) in lymphoma injected mice changed into progressive immunosuppression. To check the ability of various lymphoid cell populations to abolish this immunosuppression and restore normal reactivity, the following experiments were performed.

2. *Restoration of immunoreactivity in lymphoma-bearing mice*

2.1. *Skin allograft rejection.* Mice with lymphoma received intraperitoneal injection of 10^8 viable cells from lymph nodes of normal syngeneic mice 14 or 25 days after lymphoma transplantation and allogeneic skin graft was applied on the same day. The rejection time of allogeneic skin graft was shortened back to control value seen in normal mice, or became even shorter (Table 2). The same injection did not change the ability of normal mice to reject the allograft.

Injection of 10^8 spleen cells failed to restore immunoreactivity in lymphoma bearing mice (Table 2).

2.2 *Plaque-forming cell response.* Intraperitoneal injection of 10^8 cells from syngeneic lymph nodes, given 3 days before SRBC injection, significantly increased the number of PFC in the spleen of mice with lymphoma (Table 3). In mice having had the tumor for 14 days the PFC response was restored back to the values seen in normal mice, but in the mice having had the tumor for 25 days the PFC response was restored only partly and still remained below that in normal mice.

Similarly to the failure in restoring the skin graft rejection the injection of spleen cells from normal syngeneic donors did not reverse the decrease of the PFC values in tumor-bearing mice (Table 3).

Hence, lymph node cells, but not spleen cells from normal syngeneic donors, rescue the mice with tumor from the state of immunodepression. In order to determine the influence of environment altered by the tumor on the ability of the available immunocytes to react, the following experiment was performed.

3. *Transfer of spleen cells from tumor-bearing mice to syngeneic lethally irradiated recipients*

An injection, comprising 10^7 spleen cells from a lymphoma-bearing mouse or from a normal mouse, was given i.v. into lethally irradiated syngeneic recipients. Namely, 10^7 cells from the spleen of each donor were injected to a separate recipient. SRBC were injected 4 days before PFC assay, i.e. 3 days after transfer of spleen cells. The results are given in Table 4. Spleen cells from mice that have had lymphoma for 15 or 30 days produced significantly ($P<0.01$) higher number of PFC in the spleens of the recipients than the spleen cells from normal syngeneic donors without tumor. The difference was present even when the results were calculated in terms of the total number of splenocytes from each type of donor (lymphoma-bearing or normal) as shown in Table 4.

DISCUSSION

Recent studies have mainly focused the attention on impaired immune response to the tumor itself, both in man and in selected animal tumor models [5–9] rather than on generalized immunosuppression accompanying tumor growth.

As presented, the growth of syngeneic lymphoma in C3Hf/Bu mice was accompanied by generalized impairment of the immunological response to antigens such as SRBC (a T-cell dependent antigen) or skin allograft (which induces T-cell dependent response). In these mice a progressive depletion of lymph nodes and spleen has been expressed and the appearance of tumor cells in these lymphoid organs occurred in the late stage of the disease [9]. Our results prove that the prolongation of allograft survival time and the decrease of plaque-forming ability in lymphoma-bearing mice could be corrected by injecting lymph node cells from normal syngeneic donors. This might indicate a reduced number of lymphoid cells in tumorous host as a simple cause of the depression. But, the results of transplantation of the spleen cells from tumorous donor to lethally irradiated recipients clearly show that these donors possessed a confident number of reactive lymphoid cells.

The increased number of suppressor cells and a high level of immunosuppressive humoral factors, as blocking factor, fetal tissue antigens, normal immunoregulating proteins, are considered to be responsible for generalized immunosuppression of tumorous host [12]. These factors could compete for T lymphocytes and thus interfere with efferent limb of the immune response to tumor [5, 6]. Furthermore, the response of spleen cells to mitogenic or antigenic stimulation was suppressed if the cells had interacted with syngeneic tumor

Table 2. Survival of C57BL skin allograft on C3Hf/Bu mice bearing lymphoma for 14 and 25 days respectively and injected with lymphoid cells from normal syngeneic donor at the time of grafting

Interval after injection of tumor cells	Source of 10^8 syngeneic lymphoid cells	Number of mice	Days of allograft survival (Mean ± S.E.)	Significance (*t*-test)
14 days	Spleen	10	12.1 ± 0.5	P_1 N.S., P_2 N.S.
	Lymph nodes	10	10.8 ± 0.4	$P_1 < 0.01$, P_2 N.S.
	None	10	13.0 ± 0.6	
25 days	Spleen	12	17.6 ± 0.8	P_1 N.S., $P_2 < 0.01$
	Lymph nodes	12	11.9 ± 0.3	$P_1 < 0.01$, P_2 N.S.
	None	10	19.8 ± 0.5	
No injection (normal mice)	Spleen	11	12.2 ± 0.4	
	Lymph nodes	11	12.0 ± 0.3	
	None	10	11.8 ± 0.5	

P_1 significance to tumor bearing mice not injected with lymphoid cells.
P_2 significance to normal mice (neither tumor cells, nor lymphoid cells injection).
N.S. not significant.

Table 3. Number of PFC in the spleen of C3Hf/Bu mice bearing lymphoma for 14 and 25 days respectively, determined 7 days after injection of lymphoid cells from normal syngeneic donors

Interval after injection of tumor cells	Source of 10^8 syngeneic lymphoid cells	Number of mice	Number of PFC per spleen (Mean ± S.E.)	Significance (*t*-test)
14 days	Spleen	10	17,950 ± 1950	P_1 N.S., $P_2 < 0.01$
	Lymph nodes	12	66,340 ± 5400	$P_1 < 0.01$, P_2 N.S.
	None	12	13,100 ± 3190	
No injection	None	8	55,733 ± 1840	
25 days	Spleen	11	7365 ± 1560	P_1 N.S., $P_2 < 0.01$
	Lymph nodes	10	21,640 ± 3380	$P_1 < 0.01$, $P_2 < 0.01$
	None	11	5430 ± 1090	
No injection	None	6	49,500 ± 2100	
No injection	Spleen	10	53,400 ± 1980	
	Lymph nodes	10	54,880 ± 3460	
	None	10	50,100 ± 2320	

P_1 significance to tumor bearing mice injected with lymphoid cells.
P_2 significance to normal mice (neither tumor cells, nor lymphoid cells injection).
N.S. not significant.

Table 4. Number of PFC in the spleen of lethally irradiated C3Hf/Bu mouse, determined 7 days after injection of 10^7 spleen cells either from normal syngeneic mouse or from the mouse bearing syngeneic lymphoma for 15 or 30 days respectively

Donors of spleen cells	Number of PFC per spleen of lethally irradiated recipient (Mean ± S.E.)	Significance (*t*-test)	Number of PFC calculated per spleen of donor (Mean ± S.E.)	Significance (*t*-test)
15 days after tumor cell transplantation (10 mice)	505 ± 36	$P < 0.01$	28,126 ± 4042	$P < 0.01$
Normal mice (6 animals)	360 ± 8		8338 ± 2050	
30 days after tumor cell transplantation (12 mice)	481 ± 11	$P < 0.01$	23,880 ± 1500	$P < 0.01$
Normal mice (6 animals)	282 ± 24		9462 ± 1020	

cells *in vitro* [13]. Thus, progressive immunosuppression in tumorous host could result from direct "subversive" action of the tumor, which may be mediated through prostaglandin pathway [3, 13]. These factors do not induce antigenic competition and do not influence the serum components necessary for various functions of macrophages and other lymphoid cells; instead they affect T lymphocytes [14].

T-cells, but not B-cells, were shown to be suppressed by the syngeneic tumor and the central defect in the depression of humoral and cellular immunity in tumor-bearing host seems to be in T-cell population [3, 6]. With this in mind, normal syngeneic lymph node cells were given to tumorous host, supposing that injected cells—mostly T (T_2 cells) population—would overcome immunosuppressive factor(s) in tumorous host, and destroy allogeneic skin grafts and enhance the response of B-cells against SRBC in PFC assay. Our results fully confirmed the supposition. However, the possibility that tumor cells could be attacked following lymph node cells injection was not investigated in our system. On the other hand, the injection of spleen cells from a normal donor was not able to restore the reactivity of the tumorous host: this is probably due to the small number of T-cells (mostly T_1-cells) among the macrophages, K-cells and B-cells in the spleen.

It was further shown that the response of spleen cells to SRBC could be enhanced by the addition of T-cells educated to a second non-cross-reacting antigen, but only if the educating antigen is present [15]. This could explain initial high level of PFC to SRBC in spleen of present and other [16, 17] models of tumorous host, when the cells are educated to tumor's antigens. Later, due to the suppressive action of the tumor, these educated cells, as well as those influenced by them, were not able to react. In our system, lymphoma cells were presented in the spleen of mice bearing tumor for 25 days, but not in those bearing tumor for 15 days only [9]. As presented here, the decrease of the PFC number in the spleens of these two groups was significant to controls, being more pronounced in the 25-day group. One can speculate that progressive depletion of lymphoid cells from the spleen, combined with the immunosuppressive factor(s) existing in tumorous host, (probably potentiated by the presence of tumor cells in the spleen) is responsible for the depletion of PFC. Restoration of PFC number succeeded completely if the lymph node cells from normal donor had been injected in the mice bearing tumor for 15 days and partly in these bearing lymphoma for 25 days. However, our results proved that transfer of spleen cells from tumor-bearing host to lethally-irradiated syngeneic recipient resulted in a significantly higher PFC response to SRBC than when normal donor spleen cells have been injected. A higher number of PFC in lethally irradiated host injected with spleen cells from that donor bearing various, non-lymphoid, tumors in comparison to those injected with normal spleen cells was also observed in our laboratory (to be published). Spleen of tumorous host contained thus a satisfactory number of T and B cells to mount any immune reaction.

The action of immunosuppressive factors, existing in tumorous host, could be overcome by an additional injection of syngeneic T-cells, or diminished by removing the immunocytes from the tumor influenced environment. However, whether immunologic dysfunction in malignancies is a necessary component of neoplastic process or only (merely) an inconsequential sequele remain still a very challenging question.

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Effect of Medium Conditioned by Acute Myeloid Leukaemia Cells on Normal Marrow Colony-Forming Ability and Sensitivity to Cytosine Arabinoside*

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Abstract—*Medium conditioned by acute myeloid leukaemia cells (LCM) inhibits colony formation by normal human bone marrow cells. The degree of inhibition was related to the growth characteristics, in agar, of the leukaemic cells used to prepare the conditioned medium. Treatment with LCM also altered the sensitivity of the normal colony-forming cells to cytosine arabinoside (ara-C) in a manner proportional to the level of inhibition caused by exposure to LCM.*

INTRODUCTION

In acute myeloid leukaemia (AML) not only does the marrow contain a large population of leukaemic cells but normal haemopoietic elements are reduced. This implies a relative growth advantage to the leukaemic cells which could be due, at least in part, to a suppressive effect of these cells on the normal precursor population in the marrow. Although it is uncertain whether such an effect is operative *in vivo*, data are available which show that cells from AML patients, or medium conditioned by AML cells, inhibit the proliferation of normal bone marrow cells in agar culture *in vitro* [1–3].

The action of several drugs used in the treatment of AML, notably cytosine arabinoside (ara-C), depends on the proliferative characteristics of the target cells. Consequently, an inhibitory effect of leukaemic cells which altered the proliferation of the residual normal cells could also reduce the toxicity of the drug to normal haemopoietic tissue.

It is well known [4, 5] that cases of AML at diagnosis show different growth patterns in agar *in vitro*. Some produce colonies and/or clusters of cells while others do not grow at all. It may be speculated that leukaemic cells showing different growth patterns in culture may also exert different inhibitory effects on normal marrow cells *in vitro*. The purpose of this study was to investigate the relationship between the growth of leukaemic marrow cells in culture and their inhibitory effects on normal precursor cells both in terms of colony formation and their altered sensitivity to the drug cytosine arabinoside (ara-C).

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MATERIAL AND METHODS

Two-5 ml of marrow was aspirated from the iliac crests of AML patients at presentation or from patients with solid tumours. In the case of the solid tumour patients there was no evidence of marrow involvement by tumour and these patients were considered to be haematologically normal. A total of 21 untreated leukaemic samples and 18 normal marrow samples were used for this study.

The colony-forming ability of all marrow samples was assayed using an Agar Diffusion Chamber technique which has been described in detail elsewhere [6]. For this, 2×10^5 nucleated marrow cells, suspended in agar-medium, were introduced into diffusion chambers which were then inserted into the peritoneal cavities of irradiated (900 rad) C57Bl mice. Seven to eight days later the granulocytic colonies in the agar were counted.

Conditioned medium was prepared from both normal and leukaemic marrow cells according to the method of Chiyoda *et al.* [3]. Washed bone marrow cells were diluted to 3×10^6/ml in McCoys medium containing 15% Special Bobby Calf Serum (Gibco Bio-cult, Scotland). This suspension was incubated at

37 C for 3 days when the supernatant was obtained by centrifugation and frozen at −20°C until use (within 1 month).

Normal bone marrow cells were exposed to these conditioned media in suspension cultures: 1 ml cultures containing $2\text{–}3 \times 10^6$ normal marrow cells and varying amounts of conditioned medium were set up in McCoys Medium containing 15% serum, gassed with 10% CO_2 in air and incubated at 37°C for 3 hr. These suspension cultures were then diluted 10-fold and colony formation was assayed in Agar Diffusion Chambers. The mean control yield, following incubation without added conditioned medium, was 35.9 ± 5.2 colonies per chamber. Four chambers were scored for each experimental group so that each control group contained in excess of 100 colonies.

The sensitivity of normal bone marrow cells to cytosine arabinoside (ara-C) was measured using the method described by Gordon and Douglas [7]. After 3 hr incubation in suspension culture with conditioned media, the cells were seeded in diffusion chambers and the chambers implanted into mice. The cells within the chambers were exposed to ara-C by intravenous injection of 200 mg kg^{-1} of the drug into the host mouse. Two hours later the chambers were transferred into secondary recipients and colony formation was scored after a further 7–8 days.

RESULTS

The leukaemic cells used to condition medium for these experiments were grouped into 3 categories which were based on the maximum aggregate size achieved in agar culture in diffusion chambers. These groups were defined by (1) no growth; (2) the formation of small clusters of fewer than 30 cells; (3) the formation of large clusters (30–50 cells) and/or colonies of more than 50 cells.

Figure 1 shows the dose-dependent inhibition of normal colony formation by medium conditioned with non-growing leukaemic cells (LCM). The LCM was added to suspensions of normal marrow cells at concentrations of up to 25% and incubated for 3 hr when colony formation was assayed. Where more than one point was shown for a given concentration, different normal marrows were used for each measurement. All further experiments used a concentration of 10% LCM.

The preincubation period of 3 hr was used because the yield of colony forming cells from normal marrows incubated without the addition of CM for this length of time averaged 90% of their initial value. Longer periods of incubation resulted in a rapid reduction of colony-forming ability. Furthermore, when the marrow was seeded in diffusion chambers immediately after the addition of LCM no colony inhibition was seen, while the level of inhibition increased with the length of the preincubation period (data not given).

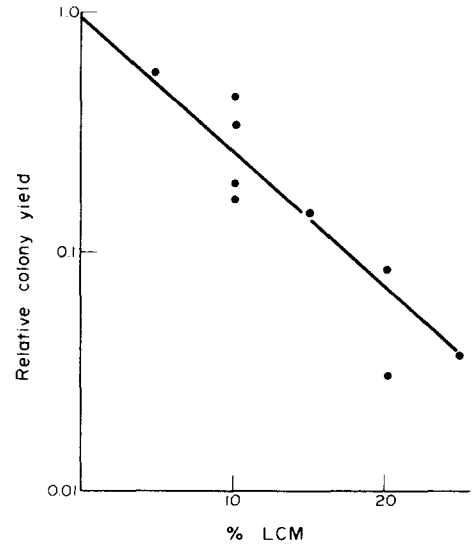

Fig. 1. Level of normal marrow inhibition as a function of the concentration of leukaemic cell conditioned medium (LCM).

Leukaemic CM derived from non-growing, small cluster forming or large cluster and colony forming leukaemias gave different levels of inhibition of normal colony formation. Medium conditioned by normal bone marrow cells had little, if any, inhibitory effect (Fig. 2). LCM from non-growing leukaemic cells gave the greatest and most consistent inhibition while inhibition by small cluster forming LCM was more variable. Medium conditioned by large cluster and colony forming leukaemic marrow was least inhibitory. The effect of large cluster and colony forming LCM was not significantly different from that of normal cells CM (Student's *t*-test). The inhibition of colony formation by non-growing LCM and small cluster forming LCM was significantly different from the controls ($P<0.001$; $P<0.01$ respectively) and from the effects of large cluster and colony forming LCM ($P<0.001$; $P<0.01$ respectively). There was, however, no significant difference between the inhibitory effects of non-growing and smaller cluster forming LCM.

Figure 2 also shows the results of repeated measurements made using individual batches of LCM against different normal marrow cells. It is evident that there was little variation

in the effect of each LCM or different target cells and no significant loss of inhibitory activity during the time between the measurements. The data in Fig. 2 was obtained within

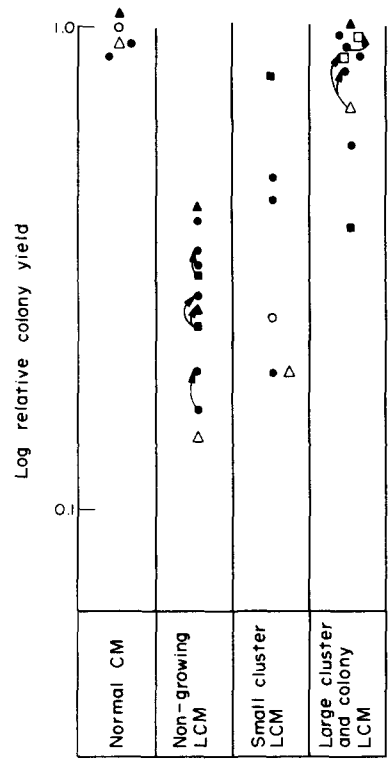

Fig. 2. The different effects of leukaemic cell conditioned medium (LCM) derived from non-growing, small cluster forming and large cluster or colony forming leukaemias, compared with the effect of medium conditioned by normal bone marrow cells. Arrows indicate where one LCM preparation was tested against different normal marrows. (●) shows where one CM preparation was assayed against an individual normal marrow. The other matched symbols show where different CM preparations were assayed against the same normal marrow.

a month of LCM preparation. Some loss of activity was detected when LCM was tested more than 2 months after it was prepared and these data are not included in the figures.

The effect of cytosine arabinoside (ara-C) on the normal cells forming colonies following incubation with LCM is shown in Fig. 3. Treatment of cells with ara-C, following incubation in suspension culture without the addition of CM, gave a survival of 35–45% of the control value. Addition of normal cell CM during the preincubation period did not alter the sensitivity of the colony forming cells to the drug. In contrast, LCM from non-growing and small cluster forming leukaemias tended to reduce the sensitivity of normal cells to ara-C while medium conditioned by large cluster and colony forming leukaemic cells tended to increase their sensitivity to the drug, although these effects could not be shown to differ significantly from the controls. However, cell sensitivity to ara-C, following incubation with large cluster and colony forming LCM was significantly different

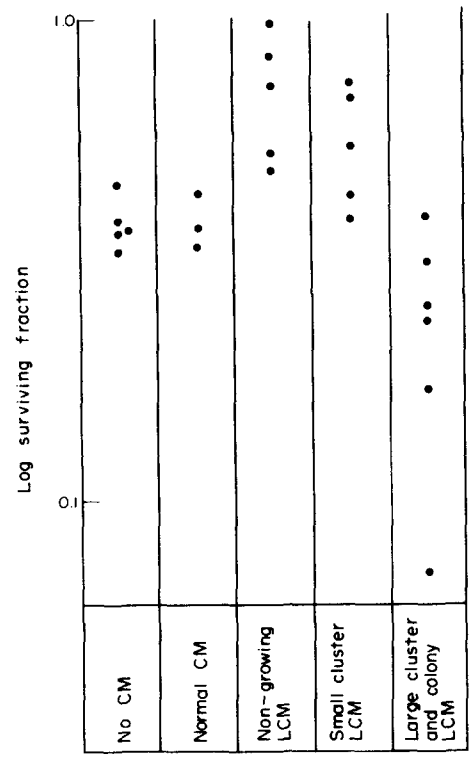

Fig. 3. Survival of cells treated with 200 mg kg^{-1} *ara-C following exposure to leukaemic or normal CM, compared with the survival of cells not exposed to CM.*

from that observed after incubation with non-growing or small cluster forming LCM ($P<0.01$; $P<0.01$ respectively).

It is apparent, from the results in Figs. 2 and 3, that the conditioned media which inhibit normal colony formation also tend to lower the sensitivity of the remaining colony forming cells to ara-C. Cells treated with less inhibitory CM show a trend towards increased drug sensitivity. These data are combined in Fig. 4, showing more clearly the relationship between the two parameters. ($r=0.79$; $P<0.001$).

DISCUSSION

The aim of cancer chemotherapy is to achieve differential cell kill between normal and tumour cells thus increasing the chances of tumour eradication without intolerable damage to normal tissue. Experimental studies have measured drug toxicities against normal and malignant cells *in vitro* with a view to selecting compounds meeting this requirement. Such studies are often made using

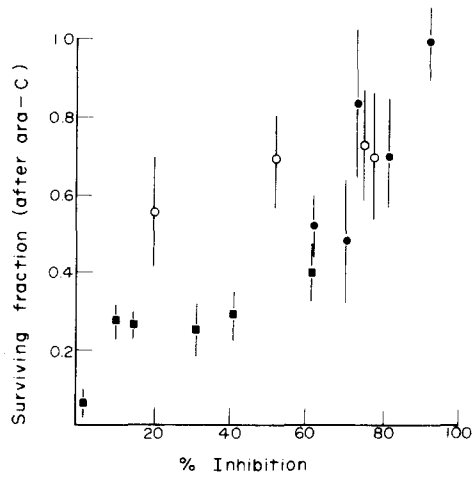

Fig. 4. Relationship between the level of normal marrow inhibition by leukaemic cell conditioned medium (LCM) and sensitivity to ara-C following exposure to LCM derived from ● non-growing, ○ small cluster forming or ■ large cluster and colony forming leukaemias.

normal animal or human cells whose sensitivity may then be compared with that of tumour cells. However, data obtained in this way can be valid only with the *caveat* that the presence of tumour does not in any way modify the drug sensitivity of normal cells.

Chiyoda *et al.* [2] and Morris, McNeill and Bridges [1] found inhibition of normal colony formation then normal marrow was cultured in the presence of leukaemic cells *in vitro*. In addition, Chiyoda *et al.* [3] measured normal colony inhibition by a homogenate of leukaemic cells, its fractionated extracts and medium conditioned by leukaemic cells. The use of conditioned medium was chosen for this study since it can be stored frozen for a limited period [3] and we were not therefore always dependent upon the availability of normal and untreated AML marrow samples at the same time.

Culture of untreated AML samples in Agar Diffusion Chambers gives similar patterns of growth to those seen in agar *in vitro* [4, 5]. The inhibitory effects on normal marrow of CM from leukaemic cells belonging to the three culture groups (no growth; small cluster forming; large cluster and colony forming) were different. Thus, although the finding that leukaemic cells inhibit normal colony formation is not new, we are not aware of any published data relating the level of inhibition to the initial growth pattern in culture.

There have been several reports of the prognostic value, in AML, of leukaemic cell culture [4, 5, 8]. Moore *et al.* [4] used the abnormal proliferation of AML cells *in vitro* to define groups of patients with good and bad prognosis. More recently, Spitzer *et al.* [5] have provided a simpler classification of *in vitro* growth and prognosis but identified different culture groups from Moore *et al.* [4]. On the other hand, Curtis *et al.* [8] found no correlation between culture findings and the rate of remission induction. Our data (unpublished), using Agar Diffusion Chambers to investigate 100 untreated AML, patients indicate that colony forming leukaemias have a lower remission rate than non-growing or cluster forming leukaemias. This is in agreement with Spitzer *et al.* [5] although Moore *et al.* [4] found a higher remission rate in colony forming leukaemias than in non-growing or cluster forming groups.

The present study has shown that normal cell inhibition by LCM depends on the ability of the leukaemic cells themselves to grow in culture. There was also a wide range of inhibitory activity within each culture group (Fig. 2). Should these ranges correlate with prognosis, such data would be useful in increasing the accuracy of prediction based on leukaemic cell culture alone. At the present time, the number of patients investigated for both growth in culture and inhibitory activity is too small to show whether the relationship between the two parameters will aid prognosis.

Of further interest is the relationship between the level of normal cell inhibition and their sensitivity to ara-C. The inhibition of normal bone marrow colony formation by factors derived from leukaemic cells suggests that the proliferative characteristics of these colony forming cells may be changed. It is, therefore, reasonable to suggest a resultant change in normal cell sensitivity to drugs, particularly those whose action depends on the proliferation rate of the cells. A correlation between inhibition, modified drug sensitivity and the clinical outcome of patients would then indicate protection of normal colony forming cells in certain leukaemic individuals during induction chemotherapy and allow more aggressive chemotherapy to be considered during the cytoreductive phase of their treatment. Although the normal cells may not regain their chemosensitivity until the leukaemic cell population had been considerably reduced, this approach would be limited in practice by the numbers of leukaemic cells required to produce the inhibitor.

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Changes in Cytotoxicity and Viability of Immune Lymphocytes after X-Irradiation*

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Abstract—*The effect of X-irradiation on cytolytic immune lymphocytes was studied* in vitro. *The splenic lymphocytes of C57BL mice immunized against mastocytoma P815-X2 cells of DBA/2J mice were irradiated and the sequential change in cytotoxicity against the tumor cells was measured by the* ^{51}Cr *release method* in vitro. *From the extent of* ^{51}Cr *release, the relative numbers of cytolytic lymphocytes (lytic unit) were then calculated. The change in viability was also measured by the trypan blue exclusion method at various time intervals after irradiation.*

A slight but statistically significant decrease in the number of lytic units was observed immediately after irradiation with doses higher than 125 rad. *The number of lytic units declined exponentially at a dose-dependent rate so that almost no cytotoxicity could be detected later than* 10 hr *post-irradiation with* 500 rad. *The shape of the dose–effect curve suggested that the cytotoxic immune lymphocytes from the spleen are heterogeneous with respect to radiosensitivity.*

The viability of lymphocytes did not change for 2 hr *after irradiation with* 75–500 rad, *but declined during* 2–7 hr *after irradiation and then slowly thereafter. A small portion of the lymphocytes was still viable* 10 hr *after irradiation with* 500 rad, *suggesting the presence of relatively radioresistant lymphocytes in the spleen.*

The variance in the rate of changes in the cytolytic function and viability of immune lymphocytes suggested that the decrease in the cytolytic ability of lymphocytes following irradiation is due to qualitative as well as quantitative changes in the immune lymphocyte population.

INTRODUCTION

CELLULAR immune reactions are mediated by thymus-derived lymphocytes (T cells), while humoral immune reactions are mediated by bone marrow-derived lymphocytes (B cells) [1–4]. In view of the frequent use of radiation to suppress host-rejection of allografts, and the extensive use of radiation for treatment of malignant tumors, a better understanding of the effect of radiation on the immune responses mediated by T and B cells is highly desirable. Although lymphocytes are generally regarded as radiosensitive [5–6], immune cytotoxic T cells have been reported to be rather radioresistant [7–10]. Ritter *et al.* [11] reported that immune C57BL anti-DBA/2 spleen cells were cytotoxic after irradiation with 50–100 rad. These investigators, however, found that the lymphocytes were no longer cytotoxic 18 hr after irradiation, even though the potential for antibody formation was still present.

In these studies the change in the total cytotoxicity of the lymphocyte population was measured. On the other hand, we investigated the sequential change in the concentration of cytotoxic immune lymphocytes at various times after various doses of irradiation *in vitro* in the present study. We also studied the change in viability of lymphocytes to determine the correlation between the change in the relative number of cytotoxic lymphocytes and the viability of splenic lymphocytes.

MATERIAL AND METHODS

The splenic lymphocytes of C57BL mice immunized against mastocytoma P815-X2 cells of DBA mice were used as immune lymphocytes. The extent of damage to the target cells by the immune lymphocytes was measured by the ^{51}Cr release method *in vitro*. This system has been used by a number of investigators and is believed to be the simplest and the most defined for the study of

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T cell-mediated cytolysis of immunogenic grafts or tumors [9, 12 20].

Immunization and lymphocyte preparation

DBA/2J mice (Jackson Laboratories, Maine) were transplanted with syngeneic P815-X2 mastocytoma cells and the ascitic tumor cells were harvested several days later. The cells were washed 3 times with RPMI 1640 medium (Associated Biomedic Systems, Buffalo, NY) and the number of viable cells was counted using the trypan blue exclusion method. About 3×10^7 cells in 0.3 ml of RPMI 1640 medium were injected i.p. into 8–10 week-old C57BL male mice (inbred in our laboratory). Control mice received the same volume of medium alone. Spleens were removed from the control and immunized mice 10 or 11 days later, minced with scissors and pressed through a stainless steel screen. The cells were left in a petri dish for 40 min at room temperature and the non-adherent cells from 3 spleens were pooled for each experiment. The lymphocytes were further purified by centrifugation on a Ficoll–Hypaque gradient [20]. After several washings, the lymphocytes were re-suspended in RPMI 1640 medium supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal calf serum (hereafter this will be referred to as complete medium) and the number of viable cells was counted. The viability at this time was about 95% and less than 5% of the viable cells were nonlymphocytes.

Irradiation

Immune and non-immune lymphocyte suspensions at 7×10^5 cells/ml were transferred to plastic culture flasks (Falcon 3013) and irradiated with 220 kV X-rays at 15 mA, using a 1 mm Al filter and a dose rate of 290 rad/min. A portion of lymphocytes was treated the same but sham-irradiated and used as non-irradiated controls.

Target tumor cells

The P815-X2 mastocytoma cells were harvested from DBA/2J mice and cultured as a suspension culture for 2–3 days in complete medium. About 2×10^6 exponentially growing tumor cells obtained from the culture were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ in 1 ml of complete medium for 40 min at 37°C with gentle rocking. The cells were then washed 3 times and resuspended in complete medium. To remove the loosely-bound ^{51}Cr from target tumor cells, the cell suspension was incubated for 20 min at 37°C and washed twice.

Cytotoxicity and viability of lymphocytes

One ml of lymphocyte suspension containing 7×10^5 cells was transferred into plastic culture tubes (Falcon 3033) immediately after irradiation, and kept stationary in a 37°C incubator gassed with humidified air containing 5% CO_2. At various times after the irradiation the viability of the lymphocytes was determined by the trypan blue exclusion test and also the cytotoxicity measured. For the determination of cytotoxicity, 1×10^5 ^{51}Cr-labeled tumor cells in 1 ml of complete medium were added to the lymphocyte suspensions and incubated for 6 hr at 37°C with gentle rocking (6 cycles/min). After 6 hr, the total radioactivity in the tubes was counted using a γ-scintillation counter. The tubes were then centrifuged at 2000 rev/min for 5 min. One ml of the supernatant was carefully transferred to another tube, mixed with 1 ml of water to equalize the counting geometry and the radioactivity counted. The percent ^{51}Cr release was calculated using the following formula:

$$\%\ ^{51}\text{Cr release} = \frac{^{51}\text{Cr activity in 1 ml supernatant} \times 2}{\text{Total } ^{51}\text{Cr activity}} \times 100$$

A number of investigators have used the ^{51}Cr activity which could be released from the target cells by freeze-thawing as the total ^{51}Cr activity for the calculation of percent ^{51}Cr release. However, we used the total ^{51}Cr activity incorporated into the target tumor cell for the calculation because we, as well as other investigators, found that the activity of ^{51}Cr recoverable following freeze-thawing lysis of target cells is sometimes variable [14, 21]. Non-immune lymphocytes were used as control in all of the experiments.

RESULTS

We first investigated the relationship between the per cent ^{51}Cr release and the ratio of the number of immune lymphocytes to the number of target cells. As shown in Fig. 1, the percent ^{51}Cr release from 1×10^5 labeled target cells increased linearly as a function of the logarithm of the number of immune lymphocytes up to 10 $\times 10^5$ lymphocytes. This relationship is in agreement with that found by other investigators [17, 21–24]. In the following experiments, 7 $\times 10^5$ immune or non-immune lymphocytes were used with 1×10^5 tumor cells in 2 ml incubation mixtures. Using immune lymphocytes under the above conditions, 64.2 $\pm$ 1.5% of the total ^{51}Cr was released from the labeled target cells in 6 hr. When corrected for the ^{51}Cr release induced by 7×10^5 non-immune

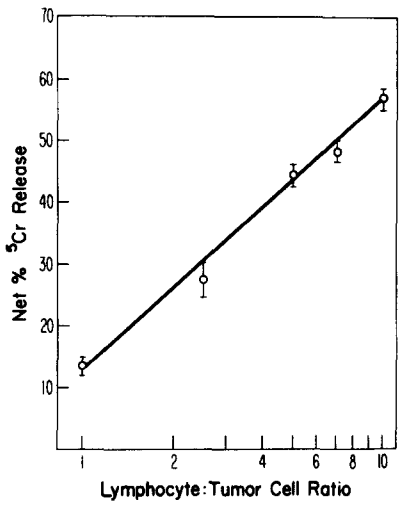

Fig. 1. Net % release of ^{51}Cr from labeled target cells as a function of immune lymphocyte to target cell ratio. Incubation mixtures containing 1×10^5 target cells and immune lymphocytes ranging from 1×10^5 to 1×10^6 cells were incubated for 6 hr, and the ^{51}Cr release was measured. The ^{51}Cr released by non-immune lymphocytes was subtracted from that released by immune lymphocytes to obtain the net ^{51}Cr release. Averages of 7 experiments with duplicate measurements and S.E. are shown.

lymphocytes, which was $15.7 \pm 0.8\%$, the net ^{51}Cr release induced by the immune lymphocytes was 48.5%. This number of immune lymphocytes was arbitrarily considered to possess 1 lytic unit [2]. Based on the net percent ^{51}Cr release from labeled target cells, the number of cytotoxic lymphocytes in each experimental lymphocyte population was graphically estimated from Fig. 1. The number of lymphocytes thus obtained was then divided by 7×10^5 to obtain the number of lytic units present.

Figure 2 shows the changes in the number of lytic units in non-irradiated and irradiated immune lymphocyte populations as a function of time. The 0-rad curve shows that the number of

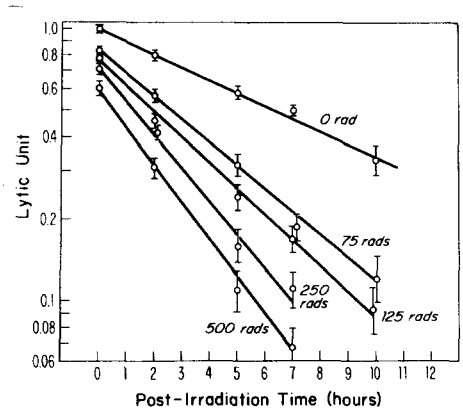

Fig. 2. The number of lytic units at various times after irradiation. Immune lymphocytes were irradiated with various doses of X-rays and the number of lytic units determined from the ^{51}Cr release. The cytotoxicity of non-irradiated immune lymphocytes (0 rad) at 0 hr is designed as one lytic unit. Averages of 10 experiments with duplicate measurements and S.E. are shown.

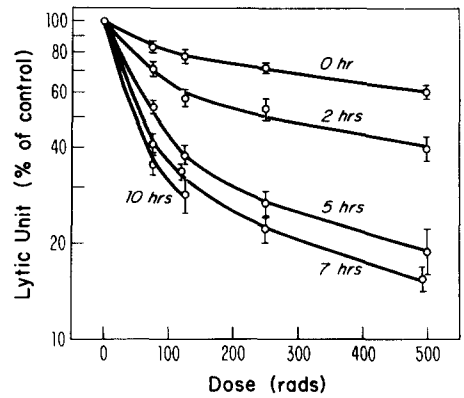

Fig. 3. Changes in lytic units as a function of irradiation dose. The numbers of lytic units at various times after irradiation were expressed as a per cent of the number of lytic units of non-irradiated lymphocytes (0-rad) at the corresponding time of measurements. Averages of 10 experiments with duplicate measurement and S.E. are shown.

lytic units of non-irradiated lymphocytes decreases exponentially as a function of time, reaching a value of 0.35 in 10 hr. The number of lytic units of lymphocytes decreased immediately after irradiation. Although the decrease was rather small, it was statistically significant after doses higher than 125 rad ($P < 0.05$). The number of lytic units of irradiated immune lymphocytes falls exponentially up to 10 hr, the extent of our observation. It appears that the higher the dose of radiation, the faster the decrease in number of lytic units. Using the same data, shown in Fig. 2, the change in number of lytic units was plotted as a function of radiation dose at various times after irradiation of lymphocytes in Fig. 3.

The number of lytic units of non-irradiated immune lymphocytes incubated for various lengths of time (0-rad curve of Fig. 2) was taken as the control value for the time and the number of lytic units of irradiated immune lymphocytes at various times was expressed as a percentage of the corresponding control. For example, in Fig. 3, the number of lytic units decreased to 60 and 15% of the unirradiated control immediately after and 7 hr after X-irradiation with 500 rad, respectively. Regardless of the time of measurement, the dose–effect curves were initially steep, up to a radiation dose of 125 rad and became less steep as the radiation dose increased, suggesting that the cytotoxicity of the immune splenic lymphocytes used here are heterogeneous with regard to sensitivity to irradiation.

Immediately after preparation, about $95.3 \pm 1.2\%$ of the non-irradiated lymphocytes were found to be viable as tested by the trypan blue exclusion test. The change in viability of immune lymphocytes as a function of time after irradiation is shown in Fig. 4. At all doses of

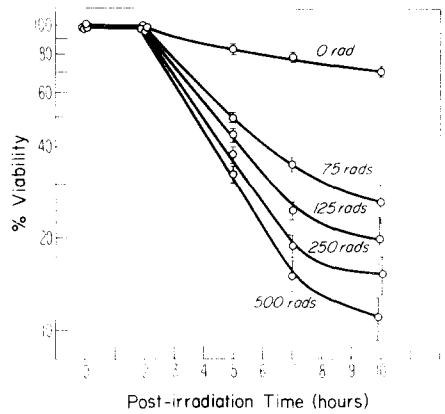

Fig. 4. Changes in viability of immune lymphocytes at various times after irradiation. The lymphocytes were irradiated with various doses of X-rays and cultured in vitro *for various time periods and the viability measured by the trypan blue exclusion method. The viability of non-irradiated* (0-rad) *lymphocytes at* 0 hr *was taken as* 100% *viable. Averages of* 10 *experiments with duplicate measurements and S.E. are shown.*

irradiation tested, there was no change in variability during the first 2 hr *in vitro*, even with a dose as high as 500 rad. The viability of non-irradiated immune lymphocytes began to decrease gradually thereafter, reaching 70% of the original value at 10 hr. The viability of irradiated lymphocytes also started to decrease after a 2-hr lag period. The rate of decrease in the viability was exponential during the 2–7 hr post-irradiation period, after which the rate of decrease became considerably slower. The rate of decrease in viability was radiation dose-dependent; the higher the radiation dose, the faster the rate of decrease. The viability of irradiated immune lymphocytes is replotted as a function of radiation dose in Fig. 5. The shape of the curves again suggests that the immune splenic lymphocytes used here are heterogeneous

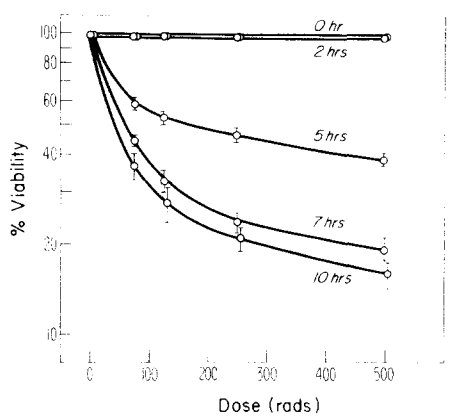

Fig. 5. Changes in viability as a function of irradiation dose. The viabilities at various times after irradiation were expressed as percent of the viabilities of non-irradiated lymphocytes (0-rad) *at the corresponding time of measurement. Averages of* 10 *experiments with duplicate measurements and S.E. are shown.*

with regard to radiosensitivity. While the majority of the cells are radiosensitive and are killed by a dose of less than 125 rad as determined in 7–10 hr after irradiation, a small proportion of the population are still viable and are thus radioresistant.

The viability of non-immune lymphocytes after irradiation was essentially the same as that of immune lymphocytes after irradiation (data not shown).

DISCUSSION

This study shows that the number of lytic units or the relative frequency of cytotoxic cells in the immune lymphocyte population decreases slightly, immediately after irradiation with doses as small as 75 rad. This decrease continues exponentially with increase in time after irradiation. This study also shows that the cytotoxic lymphocytes from spleens of mice are heterogeneous with respect to radiosensitivity.

The effect of radiation on the cytolytic activity of immune lymphocytes observed in this study may appear to be large when compared with our previous results [25] and with those of Ritter *et al.* [11]. This is due to the fact that while the changes in release of isotopes from labeled target cells was directly used to express the radiation damage on the lymphocytes in our previous report and in the study by Ritter, the ^{51}Cr release was converted to the number of lytic units in the present study because the extent of isotope-release is linearly proportional to the logarithm of the number of cytotoxic immune lymphocytes as shown in Fig. 1. For example, the net percent ^{51}Cr release by the unirradiated control immune lymphocytes was 48.5%, as described before. Immediately after irradiation of the lymphocytes with 500 rad, the net percentage of ^{51}Cr release decreased to 40.4%. This is a decrease of 16.7% [(8.1/48.5) × 100]. An analysis using Fig. 1 indicates that the decrease in net percent ^{51}Cr release from 48.5 to 40.4% is equal to a decrease in the number of lytic units from 1 to 0.6, or a decrease of 40%.

At 2 hr after irradiation (Fig. 2), the number of lytic units of irradiated lymphocytes decreased considerably while the viability of the lymphocytes remained almost intact (Fig. 4). This suggests that the initial decrease in lytic units of the lymphocyte populations after irradiation may have resulted from functional changes in the lymphocytes rather than from the death of the lymphocytes, as judged by the trypan blue exclusion test. That is, lymphocyte functions necessary for cytolytic activity such as the metabolic activity of the lymphocytes [2, 12, 13,

18, 26] or the ability to move and form aggregates with target cells [1, 12, 14, 17–19, 27–30], may be significantly damaged within 2 hr. Christensen and Jackson [31] also reported that metabolic disturbances to rat thymocytes could be observed much earlier than their decrease in viability following exposure to 1000 rad of X-rays.

A number of investigators reported that immune responses which do not require proliferation of lymphocytes are radioresistant; for example, production of migration inhibition factors (MIF) by mature T cells [32] and synthesis and secretion of antibodies by fully differentiated B cells [33]. On the other hand, immune reactions which do require proliferation of lymphocytes are radiosensitive. Katz and his colleagues [34, 35] and others [36–38] demonstrated that while the helper function of T cells can be effectively abolished when radiation is administered before T cells are differentiated, the helper function is retained when radiation is given after T cells have fully matured. The killing of target cells by immune lymphocytes does not require further proliferation of lymphocytes. Thus, it is not surprising that, in the present study, the immune lymphocytes which in time eventually die of radiation damage were still highly cytotoxic immediately after irradiation.

It has been repeatedly demonstrated that the immune lymphocytes which cause the release of ^{51}Cr from the labeled allogeneic target cells are T cells [13, 14, 16, 18, 39, 40]. Therefore, the change in the number of lytic units of splenic lymphocytes after irradiation as observed in the present study, can be attributed to the loss of functions and death of cytotoxic T cells, which constitute probably not more than 4% of the total lymphocytes of the spleen [28]. On the other hand, the decrease in viability demonstrated by the trypan blue exclusion test reflects the death of not only the cytotoxic T cells, but also of other splenic lymphocytes and macrophages, which could not be removed completely by the procedure we used. While the number of lytic units was found to decrease exponentially until 10 hr after irradiation (Fig. 2), the decrease in viability was significantly slowed down from 7 hr after irradiation (Fig. 4). This suggests that the cytotoxic T cells are more radiosensitive than other spleen cells. The shape of the dose–effect curve in Fig. 3 suggests that the cytotoxic T cells themselves are heterogeneous in radiosensitivity. Perhaps this could be attributed to the differential radiosensitivity of the cytotoxic T cells in various stages of maturation or differentiation [7, 8].

Radiotherapists are becoming more interested in the effect of radiotherapy on immunocompetence, particularly the cell-mediated immune response [41]. We feel our observation in the present study of the radiation effect on effector cells is important for the understanding of the change in host immune response against tumors in patients undergoing radiotherapy.

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Growth of a Thymoma in Diabetic Mice Treated with Insulin

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Abstract—*A transplantable thymoma of C_3H/f mice produced severe hypoglycemia in recipient mice and corrected hyperglycemia in mice with experimentally induced diabetes. Moreover, the level of the insulin content in the cell-free extract of tumour was higher than the level of insulin in sera and was very low in the muscle cell-free extract of tumour-bearing mice. The tumour grew more slowly in diabetic animals than in non-diabetic ones. However, its growth in diabetic mice treated with insulin was accelerated and resembled that in control (non-diabetic) animals. According to these data, it would seem advisable to monitor needs for insulin or other anti-diabetic medications in diabetic patients with a tumour, since this treatment may accelerate the tumour growth.*

INTRODUCTION

HYPOGLYCEMIA is observed in many animals with primary and metastatic tumours and is particularly severe in terminal stages of tumour growth [1–5]. The degree of hypoglycemia depends on the size and viability of the tumour: the larger the tumour the greater the decrease in blood glucose. Necrotic tumours are less effective in this respect [6].

A transplantable thymoma used in our laboratory [7] involves no necrosis or a minimal necrosis of the tumour tissue. It could be used, therefore, to test the working hypothesis that hypoglycemia reflects the number of live tumour cells and, for this reason, progresses with enlargement of the tumour mass. We have also investigated the influence of insulin on the rate of tumour growth and on the level of glucose in peripheral blood of mice with this transplantable tumour.

MATERIAL AND METHODS

Animals

Male C_3H/f mice, 20 weeks old, weighing about 23 g, were used in these experiments. They were obtained from the breeding colony of this Institute. Pelleted food and tap water were given *ad libitum*.

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The abbreviations used are D = diabetic, TB = thymoma-bearing, TB-D = thymoma-bearing diabetic, TB-DI = thymoma-bearing diabetic treated with insulin.

Tumour

The thymoma developed spontaneously in a C_3H/f mouse in 1970 at the M. D. Anderson Institute, Texas, U.S.A. and has been maintained in that strain by serial intramuscular transplantation of 3.5×10^6 cells into the flank. We obtained this tumour in 1972. Suspensions of tumour cells were prepared and counted by a standard technique [8]. The largest tumour diameter was recorded by caliper (Cambridge Scientific Industries, Cambridge).

Diabetes

Diabetes was induced by intravenous injection of alloxan (Merck, Darmstadt)—100 mg/kg. Hyperglycemia and glucosuria was checked 2, 8, 18 and 26 days after injection. The level of glucose in peripheral blood were determined using Hyvarinen and Nikkila's method [9]. The concentration of glucose in urine was assessed by Glukotest strips (Boehringer, Mannheim). Mice with induced diabetes and transplanted thymoma were treated daily with insulin (Pliva, Zagreb) administered intramuscularly, 8 units per mouse.

Biochemical analysis

Immunologically active insulin in sera and in cell-free-tissue extracts was determined by the two antibody method of Morgan and Lazarow [10], using porcine ^{125}I-insulin and crystalline rat insulin standards (CEA-IRE-SORIN).

Table 1. Survival of non-diabetic and diabetic mice after transplantation of thymoma and after insulin therapy

Experiment No.	Treatment	Abbreviation	No. of mice	Mean survival time (days ± S.D.)
1	Thymoma	TB	9	27.3 ± 4.2
	Alloxan and thymoma	TB-D	15	43.6 ± 2.8*
	Alloxan, thymoma and insulin	TB-DI	17	28.2 ± 6.3
2	Thymoma	TB	6	30.2 ± 4.2
	Alloxan and thymoma	TB-D	8	48.3 ± 3.9*
	Alloxan, thymoma and insulin	TB-DI	8	29.3 ± 5.9

Tumour cells were transplanted to the recipients 1 day after alloxan injection. Insulin was given daily starting 1 day after tumour implantation.
*$P < 0.05$ as compared with the control group receiving tumour cells only.

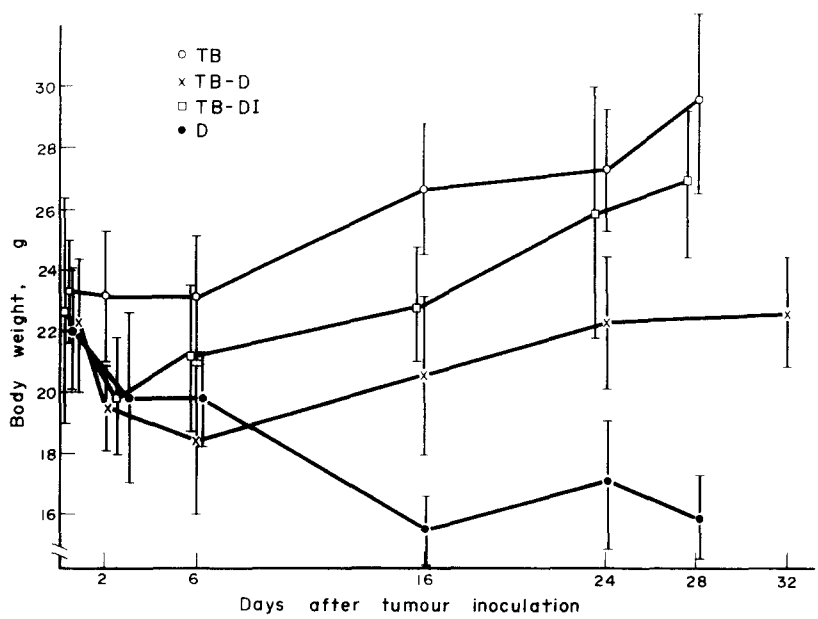

Fig. 1. Body weight (mean ± S.D.) of nondiabetic and diabetic mice after transplantation of thymoma and after insulin therapy.

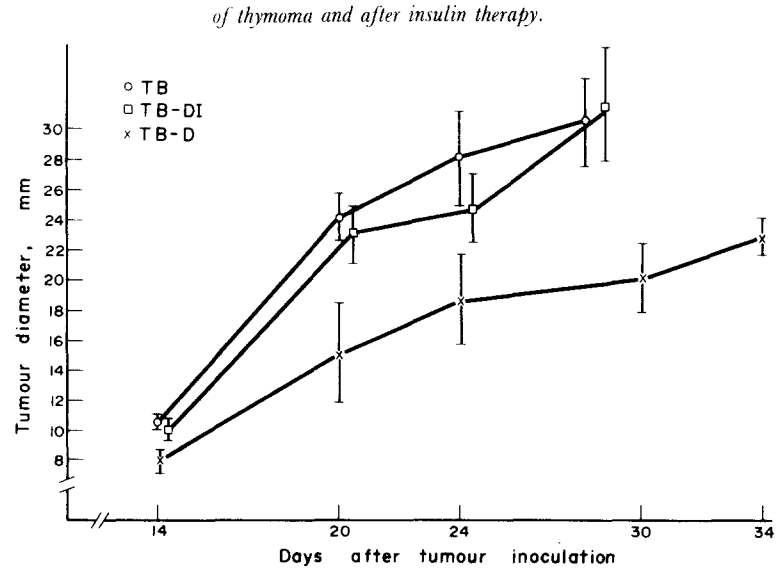

Fig. 2. Growth rate of thymoma (mean diameter ± S.D.) in diabetic mice (TB-D), diabetic mice treated with insulin (TB-DI) and in nondiabetic mice (TB); 6–8 mice per point.

Table 2. Glucose in blood (mg%) of normal and alloxan-treated mice with or without insulin

Treatment	Abbreviation	No. of mice	Days after tumour implantation 2	8	18	20	23	25
Hanks' solution	—	6			115.0 ± 16.3*			
Alloxan	D	12	561.9 ± 99.1†	487.3 ± 107.8†	471.4 ± 100.3†	—	—	579.0 ± 22.1†
Thymoma	TB	10	120.8 ± 12.8	113.1 ± 15.2	106.7 ± 16.8	95.0 ± 22.2‡	48.0 ± 12.2†‡	32.1 ± 18.6†
Alloxan and thymoma	TB-D	12	480.9 ± 105.2†	517.7 ± 57.9†	240.8 ± 20.1†	198.0 ± 33.8†‡	—	209.0 ± 137.7†
Alloxan, thymoma and insulin	TB-DI	15	213.7 ± 19.0†	128.7 ± 18.2	136.1 ± 21.1	—	100.2 ± 31.4‡	76.2 ± 48.1

Tumour cells were transplanted to the recipients 1 day after alloxan injection. Insulin was given daily starting 1 day after tumour implantation.
*The level of glucose in control mice was determined 7 days after injection of Hanks' solution.
†$P < 0.05$ as compared with the group receiving Hanks' solution.
‡4–5 mice per point.

Statistics

The results are presented as mean ± standard deviation (S.D.). Significance of differences was assessed by Student's *t*-test, the level being set at 5% ($P < 0.05$) [11].

RESULTS

Survival time of thymoma-bearing (TB) mice and thymoma-bearing diabetic mice treated with insulin (TB-DI) was approximately the same (28 days). However, the survival time of tumour-bearing diabetic mice without insulin (TB-D) was significantly longer, that is 44 days (Table 1).

The body weight varied during the tumour growth and was higher in TB and TB-DI mice than in TB-D mice and in diabetic mice without tumour (D) (Fig. 1). TB-D mice lost some weight at the onset of the experiment, but later regained part of the loss. Their weight remained somewhat lower than of TB and the TB-DI mice. The body weight of diabetic mice (D) fell continuously throughout the experimental period, to about two-thirds of the initial value after 28 days.

Tumours grew more slowly in TB-D mice than in TB and TB-DI (Fig. 2). There were no differences in the rate of tumour growth between TB mice and TB-DI mice. In other words, insulin treatment of diabetic mice "restored" the tumour growth rate back to the level seen in non-diabetic controls.

Glucose in peripheral blood was measured 2, 8, 18 and 25 days after injection of tumour cells. At the onset, the level of glucose in peripheral blood of TB mice resembled that in control mice treated with Hanks' solution, but later declined to as low as 32 mg% (Table 2). Diabetic mice with tumour (TB-D) had an initially high level of glucose in blood (480–518 mg%), similar to that maintained in diabetic mice without tumour (D) (470–580 mg%), but 18 days after tumour transplantation the level decreased to half of the initial value. Only the diabetic mice without tumour maintained a high level of glucose throughout the experiment (470–580 mg%). The treatment with insulin decreased the glucose level in diabetic mice with tumour, but these mice (TB-DI) still had higher concentrations of glucose in peripheral blood than TB mice (76–214 mg% vs 32–120 mg%).

Sixteen days after transplantation of the tumour, the level of insulin was higher in the sera of tumour-bearing mice than in control mice without tumour (Fig. 3). However, in the terminal phase of the disease the level of insulin became rather low, about 60% of the values in

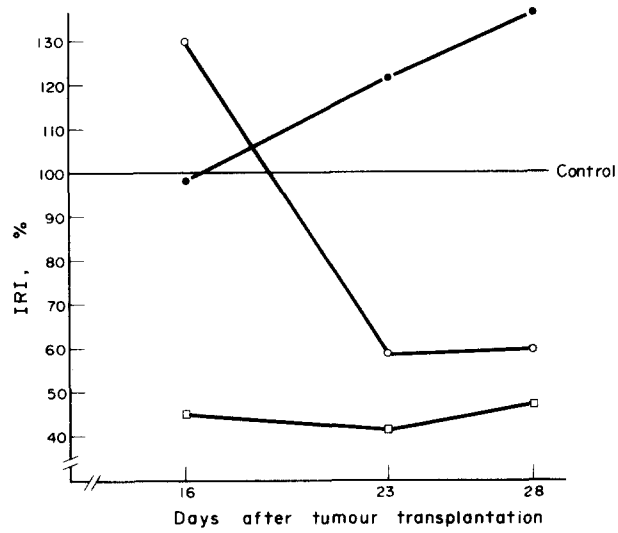

Fig. 3. Mean Insulin concentration in the sera of mice without tumour (control, n = 8), in the sera of thymoma-bearing mice (○, n = 10), in the cell-free extract of thymoma (●, n = 5) and in the cell-free extract of the muscle (□, n = 5) of thymoma-bearing mice. Concentrations of insulin have been expressed as percents of the concentration in sera of control mice.

mice without tumour. We also found that the concentration of IRI in the cell-free extract of tumour tissue was significantly higher than in cell-free extract of the muscle of the same mice.

DISCUSSION

Present studies indicate that transplantable thymoma grew faster in non-diabetic mice (TB) and in diabetic mice treated with insulin (TB-DI), than in diabetic mice deprived of insulin (TB-D). In consequence, TB-D mice survived longer than mice from the former two groups.

Diabetic animals without tumour (D) lost weight, while the other groups bearing the tumour, whether diabetic or not, gained weight. Thus, the weight gain can be attributed to the tumour. The concentration of glucose in the peripheral blood of TB-D mice decreased during the experimental period and reached half of the initial level at the end of the experiment. Hypoglycemia was particularly conspicuous in terminal stages of tumour growth. This is in agreement with other experimental studies which have demonstrated hypoglycemia in animals with tumours [1–5]. Perhaps the membranes of tumour cells are more permeable to glucose than membranes of normal cells, so that the tumour "consumes" much glucose and thus causes hypoglycemia. Indeed, many tumours contain high levels of lactic acid [6, 12] which might indicate extensive glycolisis in tumour cells. In some experimental tumours caused by viruses, transport of glucose [13] and hexose [14] into transformed cells was found to be faster and this was attributed to changes of the tumour cell membrane [13].

An alternative explanation for hypoglycemia during the tumour growth would be that the tumour secreted insulin or insulin-like substances which promoted the transport of glucose into the tumour cells [15, 16]. Another source of insulin required for that purpose could be the host. Since diabetic tumour-bearing mice do not have sufficient amounts of insulin, their tumours cannot grow as fast as in normal healthy mice. Exogenous supply of insulin would then, obviously, promote tumour growth.

We found that thymoma-bearing mice were hypoinsulinemic. This could be due to the very fast growth of tumour cells and to the fact that in the tumour cell-free extract we found significantly higher values than in muscle-cell-free extract of tumour-bearing mice. These data did not exclude the possibility that thymoma-cells produced insulin or insulin-like substances.

Hypoglycemia observed in TB-DI mice in first stages of tumour growth progressed during further growth of the tumour. This may indicate that here, also, tumour cells consumed glucose and, therefore, diminished the glucose level in the peripheral blood. Since the tumour growth was faster and survival time shorter in the TB-DI mice than in TB-D, it seems that tumours require adequate supply of insulin for their growth.

Glucagon deficiency should also be taken into account. Indeed, an acquired form of glucagon

deficiency has been found in certain human cancers [16]. In our future experiments, we shall check this parameter as well.

According to these data, a note of caution might be considered in regard to the therapy of patients with both diabetes mellitus and tumour. Diabetic patients with tumour might need monitoring as to their needs for insulin or other anti-diabetic medication, since such treatment may accelerate tumour growth, perhaps by promoting uptake of glucose into tumour cells.

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Parameter Analysis of Gompertzian Function Growth Model in Clinical Tumors

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Abstract—*In analysis of clinical tumors a conventional exponential growth model is often insufficient in clinical practice. Gompertzian function growth model is one of the practical alternatives. The way to obtain the parameters of the function is discussed with application to tumors in clinical practice. The function can be extrapolated forward and backward, which is one of the major points of interest in the practice of malignant therapy. Tumor characteristics differences are also discussed in relation to the growth parameters.*

INTRODUCTION

LAIRD [1], based on her empirical observations and an analysis of literature, proposed a Gompertzian function to tumor growth model. The idea of this model is that a tumor grows exponentially but the growth rate declines exponentially, which results in an asymptote of the tumor volume.

Clinically the mathematical function for a tumor growth model needs to be practical and simple, both are fulfilled by a Gompertzian function model, although there are some criticisms [2]. Whether this function is appreciable to human tumors is uncertain. Continuous slowing of human tumor growth rate has been reported [3] and possible existence of a maximum tumor volume has been observed, at least, in breast carcinoma skin metastases [4]. As a Gompertzian growth model comprises the exponential growth, this method of analysis can also be applied to an exponential growth. The function has been applied in this study to clinical tumors in order to obtain the values of the growth parameters.

Gompertzian function is explained by Laird [5] as "exponential growth process limited by exponential retardation", which means that growth is essentially exponential but the growth rate is exponentially reduced. This can be expressed mathematically as follows.

$$dV/dT = A^*V \quad (1)$$

$$dA/dT = -R^*A \quad (2)$$

where V stands for tumor volume, A for growth rate, R for retardation factor. These two equations lead to

$$V = Vi^* \exp[Ai/R^*(1-\exp[R^*T])] \quad (3)$$

where Vi and Ai are tumor volume and growth rate at the beginning of the observation, respectively. Equation (3) implies the following three factors.

1. *Retardation of growth rate*

In equation (3) Ai is the initial growth rate which decreases according to the following equation.

$$A = Ai^* \exp(-R^*T) \quad (4)$$

And this growth rate (A) is easily obtained by measuring the tumor at least twice within a short interval. Since

$$A = dV/dT/V \doteqdot (\log V_1 - \log V_2)/(T_1 - T_2) \quad (5)$$

log = natural logarithm.

This growth rate is only the initial value which would regress soon, as has been observed in skin metastases from breast carcinoma.

2. *Maximum volume*

Tumor does not grow beyond a certain volume which is the asymptote of the equation. (Maximum volume (asymptote volume) is symbolized by Vm). As discussed below, various reasons have been reported which account for the limitation of tumor growth. The continuous retarding effect produces the limitation of tumor

size and the maximum volume is:

$$Vm = Vi^* \exp(Ai/R) \tag{6}$$

In tumor treatment it would be convenient to know the maximum size of each tumor type. The maximum size is not only dependent on the pathological type of the tumor. Equation (6) indicates that the maximum volume also varies according to the value of R which would be influenced by the tumor environment, for example, by the blood supply.

3. *Growth rate and volume*

Equation (3) describes the uninterrupted tumor growth. When the tumor growth is disturbed, for example, when half of the tumor is excised, how does the maximum size change? Equation (3) itself does not answer this question. Todaro *et al.* [6] reported that partial removal of cells from a contact inhibited culture of normal cells induces abundant mitosis. Breur [7] described a case of mammary carcinoma with a doubling time of about 700 days which was treated by a course of chemotherapy and the mass regressed remarkably but considerably increased its growth rate. Volume dependency of growth rate is a widely accepted fact and has been discussed [8]. From equations (3) and (4).

$$A - Ai = -R^* \log(V/Vi)$$

Replacing Vi with Vm, then $Ai = 0$

$$A = -R^* \log(V/Vm) \tag{7}$$

The term $\log(V/Vm)$ must be the indicator of cell population pressure in tumor. Hence mathematically this growth model function is expressed in the following two equations.

$$A = -R^* \log(V/Vm)$$
$$dV/dT = A^* V$$

MATERIAL AND METHODS

A. *Method*

Growth parameters. For quantification of tumor therapy, it is required to obtain the values of parameters in Gompertzian function. (The computations are shown in Appendix employing at least three sets of data.)

for R

$$\frac{\log V_1 - \log V_2}{\log V_3 - \log V_2} = \frac{\exp(-R^*(T_1 - T_2)) - 1}{\exp(-R^*(T_3 - T_2)) - 1} \tag{8}$$

for Vm

$$\frac{\log V_1 - \log Vm}{\log V_2 - \log Vm} = \frac{T_1 - T_2}{T_3 - T_2} * \log\left(\frac{\log V_3 - \log Vm}{\log V_2 - \log Vm}\right) \tag{9}$$

These formulae are too complicated and a computer is required to obtain the values.

The practical approximate values can be obtained using a semi-logarithmic scale. Equations (5) and (7) are employed. The measurements usually involve considerable errors, therefore, three sets of measurements are preferable to obtain a differential value of equation (5) as is shown on the curve in Fig. 1A.

In order to avoid the extreme variation, three adjacent plots are used to obtain the gradient. The middle points of two adjacent plots are connected successively. The gradient is read using the doubling time of this line segment. The middle point of the line segment is supposed to be the corresponding tumor volume. The interrelationship between the growth rate and the doubling time is as follows:

$$A = \log 2/Td \tag{10}$$

where A is growth rate and Td for the doubling time. For example (see the curve in Fig. 1A), upper part has a doubling time of 47 days which is supposed to be the doubling time of the volume 305 mm^3. From equation (10) the growth rate is calculated and is 0.0148/day. Lower part has a doubling time of 15 days which is supposed to be that of 135 mm^3 and the growth rate is 0.0463/day.

The derived growth rates are plotted on a semi-logarithmic scale as shown in Figs. 2 and 3. The slope of this straight line is the value of retardation factor (R). However, as decimal semi-logarithmic scale is used, equation (7) has to be changed into decimal–logarithmic,

$$A = -2.3^* R^* \log_{10}(V/Vm) \tag{11}$$

The point where this straight line crosses the abscissa is the maximum volume of the tumor (Vm). For example in Fig. 2,

$$R = 0.0149/\text{day and}$$
$$Vm = 1480\ mm^3$$

Extrapolation. The use of the initial growth rate when a tumor consists of a single malignant cell raises the following hypothetical questions.

1. Whether or not it is justified to extrapolate this model to so far backward [9]?
2. Whether or not a single malignant cell is able to originate the process of a malignant tumor [10]?

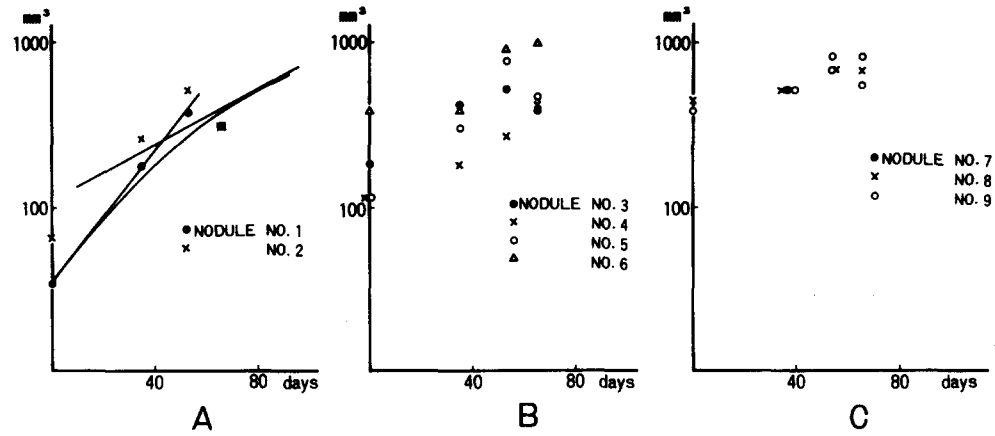

Fig. 1. Measured volumes of breast carcinoma skin metastases. The nodules are grouped according to their general sizes. A. Smaller nodules; B. Intermittent nodules; C. Larger nodules. Smaller nodules show greater growth rate. The lines in Portion A illustrate the method to obtain the doubling time of each nodule from adjacent three measurements.

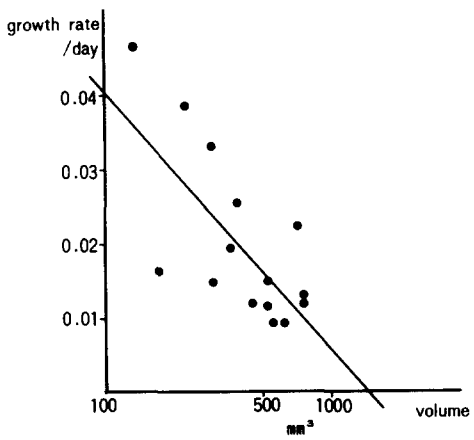

Fig. 2. Growth rate vs logarithm of volume in breast carcinoma skin metastases. Regression line $A = -0.0148^* \log(V/1480)$. *(Significant with* $P = 0.05$ *in analysis of variance.)*

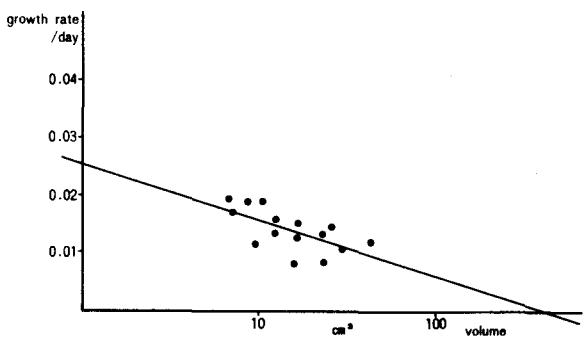

Fig. 3. Growth rate vs logarithm of volume in lung metastases from uterine leiomyosarcoma. (Abscissa in logarithmic scale.) Regression line $A = 0.00423^* \log(V/402)$. *(Significant with* $P = 0.05$ *in analysis of variance.)*

Hence it is practical to employ the possible smallest tumor size that is clinically detectable. Tumors smaller than one cubic millimeter are usually subclinical, therefore, growth rate is preferably standardized at the volume of one cubic millimeter (symbolized *As*).

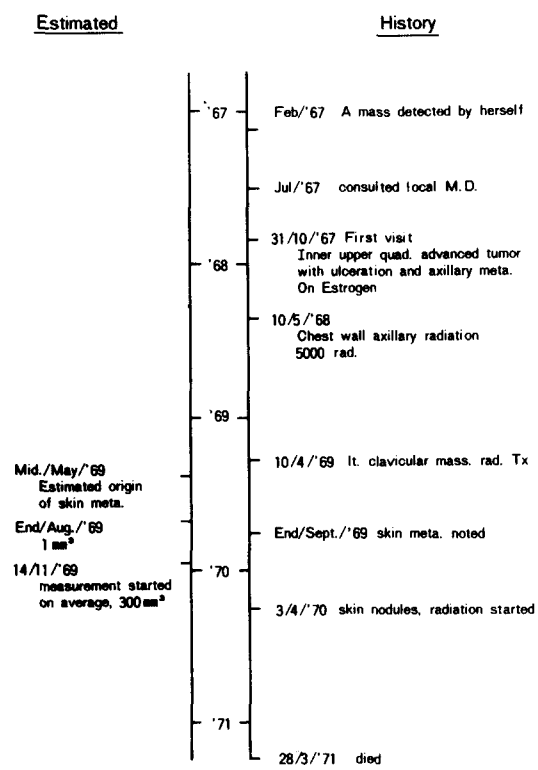

Fig. 4. Illustration of the clinical history in case 1. 72 yr *old female left breast intraductal carcinoma, T3N3M1. The estimated origin of skin metastases is in the middle of May* 1969 *and the nodules grew up to* 1 mm^3 *in a short period.*

B. *Illustrative patients*

Cautions have been taken during the measurements to avoid inaccuracy. Yet as the data have come from clinical measurements they will still contain errors.

Case 1. Figure 1 shows the measured growth curves of skin metastases from a breast carcinoma. The clinical course is illustrated in Fig. 4. The volumes of each metastatic nodule are

Table 1. *Volume change of skin metastases from breast carcinoma*

Nodule number		1	2	3	4	5	6	7	8	9
Days after initial observation	0 days	33.5	65.4	180	113	113	381	382	448	448
	35 days	180	268	402	180	301	381	523	523	523
	53 days	382	523	523	267	760	904	696	697	823
	65 days	301	301	382	423	466	979	551	697	823

Table 2. *Doubling time and growth rate change of the skin metastases*

No.		1	2	3	4	5	6	7	8	9
From the first 3 points	*V*	135	225	358	170	293	445	520	545	745
	Td	15	18	36	43	21	59	61	72	53
	A	0.0463	0.0386	0.0193	0.0162	0.0331	0.0118	0.0114	0.00965	0.0131
From the last 3 points	*V*	305	390	458	275	518	700	610	655	740
	Td	47	262	−217	24	46	18		74	58
	A	0.0148	0.00265	−0.00318	0.0261	0.0151	0.0223	0	0.00938	0.0120

V=Volume in mm^3; A=growth rate in day; Td=doubling time in days.

calculated as a rotating ellipsoid around the longer diameter measuring right angle longer and shorter diameters of a nodule. The computed volumes of this case are shown in Table 1 and are plotted on semi-logarithmic scale in Fig. 1 divided into three groups A, B and C simply according to their general sizes. The tumors are observed for 65 days and then treated. (The curves are drawn so that they go through the middle point of the two adjacent points or parallel to the line which the two points form.)

All nodules except Nos. 4 and 6 show the slowing of growth rate which the Gompertzian function predicts. In particular the three nodules in the largest group have strong tendency to flatten as if they are close to the maximum volume of these tumors. Table 2 shows the graphically measured doubling times of lower and upper portion of each curve. The mean doubling time of each volume ranges:

100–300 mm^3 is 24 days

300–500 mm^3, 47 days and

> 500 mm^3, 55 days.

(Two very large and one negative doubling times are excluded.) These observations above indicate that growth rate is volume dependent and the small nodules can be considered growing with the growth rate of the portion A of Fig. 5. (Gompertzian curve calculated with $R=0.0149$ and $A=0.1092$ at one mm^3.) The large nodules grow with a growth rate of the portion C (the straight lines A, B, C in Fig. 5 indicate the inclinations of doubling times 24, 47 and 55 days).

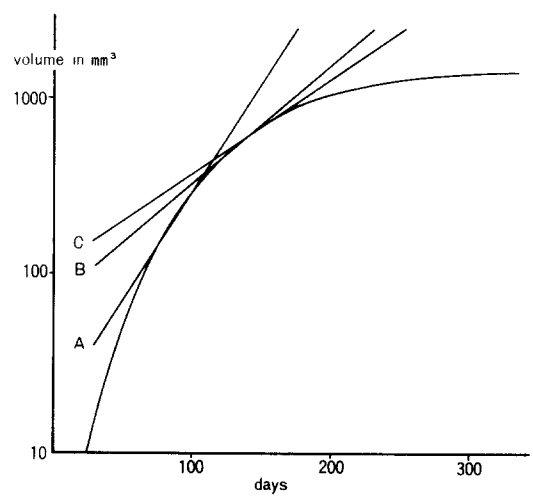

Fig. 5. *Gompertzian growth curve with parameters obtained from the data in Fig.* 1. *Lines A, B and C are the growth lines of nodules in Fig.* 1*A*, 1*B and* 1*C respectively.*

Case 2. The case has multiple lung metastases from a leiomyosarcoma of uterus. The clinical course is illustrated in Fig. 6. Two years prior to her initial visit to the clinic the patient underwent total hysterectomy and subsequently multiple courses of radiation and chemotherapy. During the observations the patient received radiation to abdomen. Sequential chest X-rays were taken and the sizes of the lung metastases measured. At the end of observation the patient was given another course of chemotherapy.

The computed tumor volumes are shown in Table 3 and graphically in Fig. 7 dividing into

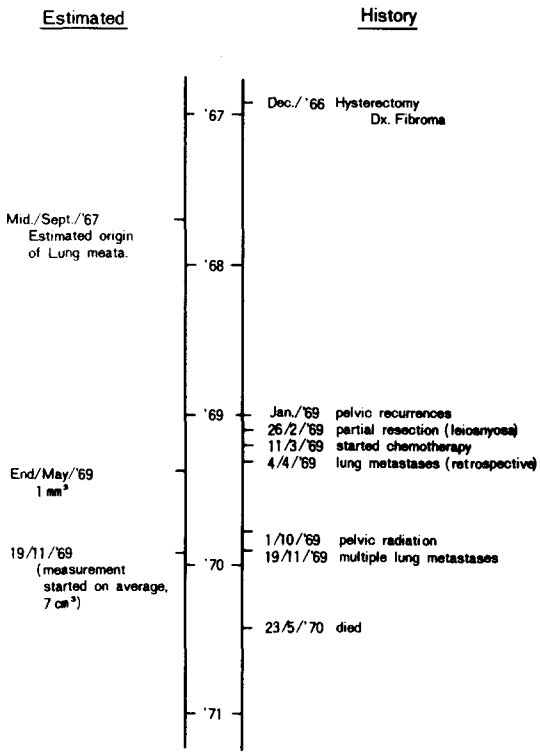

Fig. 6. Illustration of the clinical history in case 2. 42 yr old female leiomyosarcoma of uterus. The estimated origin of lung metastases is in the middle of September 1967 and they grew slowly.

Table 3. Volume change of leiomyosarcoma lung metastases

X*	0	63	94	116
1	10.3	26.5	36.1	50.9
2	8.2	18.8	26.5	33.5
3	4.8	15.6	20.6	26.5
4	3.4	14.1	20.6	32.6
5	5.5	11.5	14.1	17.1
6	1.15	4.85	9.20	17.1
7	1.15	3.97	6.59	8.44
8	2.57	6.37	8.18	9.20
9	2.87	4.8	6.08	8.50
10	1.15	1.77	2.57	3.59
11	0.904	1.24	2.14	3.59
12	0.381	0.575	0.904	1.15

*Observation day.
X No. of lung metastases unit cm^3.

three groups according to general sizes of lung lesions. Despite of the long observation period, the curves of Fig. 7 show little flattening, suggesting that the effect from retardation factor is little. Convexity downward of curves in Fig. 7(A) may be due to measurement errors or to the progression of smaller tumors [11]. Tumors in the lungs are accompanied with surrounding inflammation which are difficult to identify in the X-ray films and give us inaccurate measurements when the tumors are small. The data below an approximate volume of 6 cm^3 had to be excluded to obtain significant values, therefore, only the data of Fig. 7(C) were used in the computation.

Table 4 shows the graphically measured doubling times. (The first and last row show the doubling times obtained from the first and last two sets of measurements in Fig. 7(C), otherwise the doubling times are obtained as explained in Material and Methods.) Table 4 indicates that the mean doubling times of tumors—

$< 10\,cm^3$ is 45 days

10–20 cm^3, 50 days and

$> 20\,cm^3$, 56 days.

This change in doubling time suggests that Gompertzian function is still applicable to this case.

As a rule every metastatic nodule grows independently and each can be expected to follow the same growth curve. So if multiple lesions are available only two sets of measurements enable us to obtain the values of retardation factor and maximum volume. The values of Tables 2 and 4 are plotted on Figs. 2 and 3.

RESULTS

Applying least square law the regression lines of Figs. 2 and 3 are obtained which are the lines of equation (7). And results of the calculations are:

Case 1

$R = 0.0149$/day

$Vm = 1480\,mm^3$

$As = 0.1088$/day

$Tdo = 53$ hr

Case 2

$R = 0.00423$/day

$Vm = 4.02 \times 10^5\,mm^3$

$As = 0.0351$/day

$Tdo = 6.71$ days

One cubic millimeter of tumor contains 10^6 cells, therefore, the original doubling times at one cell (Tdo) are in Case 1, 53 hr and in Case 2, 6.71 days which are not far from the range of reported doubling times in cell cultures [12]. Figure 5 is the computed Gompertzian function curve of Case 1 with $R = 0.0149$/day and $Vm = 1480\,mm^3$. The figure shows exact curving of

Table 4. Doubling time and growth rate change of the lung lesions

No. in order of tumor		1	2	3	4	5
From the last 2 points	*V*	42.5	29.5	22.8	25.2	15.8
	Td	59	65	82	48	87
	A	0.0118	0.0107	0.00845	0.0145	0.00785
From the middle 3 points	*V*	22.5	16.3	12.3	10.5	9.5
	Td	53	56	44	37	61
	A	0.0131	0.0124	0.0157	0.0188	0.0114
From the first 2 points	*V*	16.5	12.2	8.6	6.8	7.0
	Td	46	52	37	31	40
	A	0.0151	0.0133	0.0188	0.0193	0.0173

V = Volume of tumors in cm^3; Td = doubling time; A = growth rate.

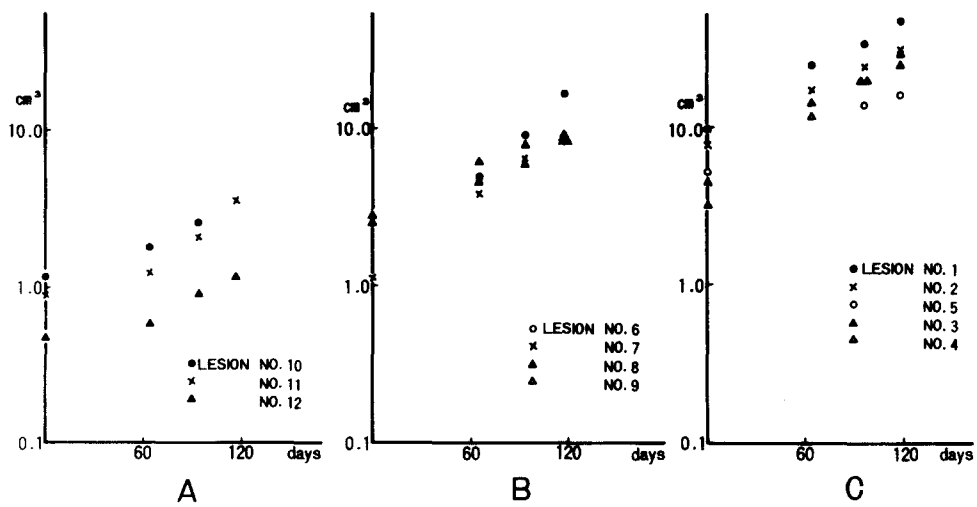

Fig. 7. Measured volumes of lung metastases from uterine leiomyosarcoma. The lesions are grouped according to their general size. A. Smaller lesions; B. Intermittent lesions; C. Larger lesions. The growth rate does not appear to change according to their volumes.

calculated doubling times from Table 2. The reported cell cycle times in cell cultures range from 10 to 40 hr. Our computed original doubling time is close to this range.

It is also interesting to extrapolate the growth curves, that is to know how long ago the tumors originated and how soon they will come to the steady phase. Using equations (4) and (7) in Case 1 the time was calculated in which a 200 mm^3 tumor reaches 90% of its asymptote volume. The result is 198 days. In Case 2 a 7 cm^3 tumor took 863 days more to reach the steady phase which is postulated here above 90% of asymptote volume. The time which these tumors required to grow from one cell to the size of 1 mm^3 is also calculated using equations (4)

Case 1 = 71 days and

Case 2 = 255 days.

Figure 5 which is the Gompertzian function curve with the parameters of Case 1 shows that a nodule of average size (300 mm^3) was the size of 1 mm^3 14.5 weeks prior to the initial observation. Ten weeks prior to this size the nodule started to proliferate. A single 3 mm skin nodule had been noticed 7 weeks before the initial measurement, which is predicted approximately from this curve. In Case 2, the computation states it takes 528 days for the leiomyosarcoma to grow from one cell to 7 cm^3 a retrospective survey of chest X-rays showed the first metastasis 150 days prior to the initial measurement. The calculation using equations (4) and (7) reveals that this tumor of 0.2 cm^3 requires 149 days to attain 7 cm^3 which is about the average size at the time of first measurement. This result suggests that these metastatic tumors were in average of 0.2 cm^3 when the first two lung lesions

were detected on her chest X-ray, which were approximately 5 mm in diameter. But it is presumed the initial doubling time of this tumor 6.7 days, which is a little longer than the reported cell cycle time, is due to the chemotherapy and is not the real initial doubling time.

DISCUSSION

There is a possibility that human tumor growth does not follow a Gompertzian function, but grows exponential. This method of parameter analysis of Gompertzian function can be applied to human tumors if they follow an exponential function growth, because exponential function is a special form of this function where the factor of R is zero. Therefore, the growth rate is constant and the regression line in the Fig. 2 has to be parallel to abscissa. Figure 7 appears that the growth rates are not retarded. But the analysis in detail in Fig. 3 did not show the parallel regression line to abscissa.

So long as the tumor growth model of Gompertzian function was inferred according to empirical observations [1] it lacked biological theoretical background, particularly in the assumption that growth rate declines exponentially. Slowing of growth rate has been explained by decreasing growth fraction and by increasing cell death [13]. In cases of skin squamous cell carcinomas, cell death rate goes up to 90% [14]. Although there is still criticism of this model e.g. it ignores tumor progression [11], it is shown here this function fits practically to tumor growth curves over a wide time scale and the extrapolation of this function gives us reasonable values.

Extrapolation forward predicts how large the tumor will grow and what is its probable mean maximum volume. For example in Fig. 5, these adenocarcinoma metastases in the skin have the mean maximum volume of 1480 mm^3 and it takes six more months that these metastases become a nearly steady state. In Case 2, a 402 g tumor in a lung frequently causes serious symptoms. Even slow growing tumors require to be treated when the significantly large maximum volume is expected. Clinically, measurement of retardation constant is important.

To solve problems in tumor treatments, such as whether the metastases originated before or after surgery, this function has to be extrapolated backward. The calculation with these cases yielded reasonable values for the backward extrapolation and suggested good clinical applicability.

Laird [1] mentioned that stimulation of natural growth retardation factors might be of practical importance in reducing the incidence of clinical malignancy. What causes the retardation is still not clear. Laird [15] states it is due to genetically programmed cell death. Besides, it can be considered that factors which make constant cell loss or differentiation of proliferating cells affect also retardation factor (R), e.g. hormone therapy, chemotherapy or irradiation. So the effect of treatments can be evaluated measuring retardation factor (R). Cell contact can be one of the factors in tumor growth retardation. It is known, cell contact is an efficient method of cell communication [16]. Immunological relation of host and tumor is also a factor of retardation. Retardation factor can be considered variable according to tumor host relationship. So constant observation is required even in the cases which have "steady" tumors, particularly in the cases that "steady" tumors are kept with treatmenrs.

Slight stimulation can cause the decrease of R too. Hell and Cruickshank [17] reported that a slight wound on skin causes remarkable increase of tritiated thymidine uptake among the cells surrounding the wound and the severe wound causes an even greater uptake.

CONCLUSION

Gompertzian function growth model appears complicated but the parameters can be obtained without difficulty in the cases of multiple lesions. Extrapolation of this model forwards and backwards is possible with these parameters with clinical accuracy. The sample cases indicate that skin metastatic adenocarcinoma from breast has a strong tendency of growth retardation and has restricted tumor maximum size. Whereas leiomyosarcoma has hardly any growth retardation and has enormous tumor maximum size.

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APPENDIX

From equations (5) and (7)

$$-R^*\log(V/Vm)=Ai^*\exp(-R^*T)$$

(21) $\log V_1-\log Vm=-Ai/R^*\exp(-R^*T_1)$

(22) $\log V_2-\log Vm=-Ai/R^*\exp(-R^*T_2)$

(23) $\log V_3-\log Vm=-Ai/R^*\exp(-R^*T_3)$

For R,

(21)–(22)

(24) $\log V_1-\log V_2=-Ai/R^*\{\exp(-R^*T_1)-\exp(-R^*T_2)\}$

(23)–(22)

(25) $\log V_3-\log V_2=-Ai/R^*\{\exp(-R^*T_3)-\exp(-R^*T_2)\}$

(24)/(25)

(8) $$\frac{\log V_1-\log V_2}{\log V_3-\log V_2}=\frac{\exp\{-R^*(T_1-T_2)\}-1}{\exp\{-R^*(T_3-T_2)\}-1}$$

For Vm,

(21)/(22) $$\frac{\log V_1-\log Vm}{\log V_2-\log Vm}=\exp\{-R^*(T_1-T_2)\}$$

(26) $$\log\frac{\log V_1-\log Vm}{\log V_2-\log Vm}=-R^*(T_1-T_2)$$

(23)/(22) $$\frac{\log V_3-\log Vm}{\log V_2-\log Vm}=\exp\{-R^*(T_3-T_2)\}$$

(27) $$\log\frac{\log V_3-\log Vm}{\log V_2-\log Vm}=-R^*(T_3-T_2)$$

(26)/(27)

(9) $$\log\frac{\log V_1-\log Vm}{\log V_2-\log Vm}=\frac{T_1-T_2}{T_3-T_2}*\log\frac{\log V_2-\log Vm}{\log V_2-\log Vm}$$

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Purification of an Inhibitor of DNA Synthesis in Mammary Cells*

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Abstract—*A small molecular weight inhibitor of DNA synthesis, specific for normal and malignant mammary cells but devoid of species-specificity, has been isolated from bovine mammary glands; this factor has little effect on the overall RNA metabolism.*

INTRODUCTION

CELLULAR multiplication appears to be controlled by stimulatory and inhibitory factors. However, so far, in the case of the mammary epithelium, attention has been focused only on hormones acting as stimulatory factors. Some studies have been done *in vitro*, and insulin, the epidermal growth factor, estrogens [1] and a serum factor [2] were shown to stimulate DNA synthesis in mammary cells.

We have previously reported the isolation from bovine mammary glands of a small molecular weight inhibitor of DNA synthesis in normal and malignant mammary cells; this factor had no gross effect on RNA metabolism [3]. In this paper, we describe the method of purification of this inhibitor.

MATERIALS AND METHODS

A. *Solutions*

Hanks' solution. To 100 ml of 10-fold diluted Gibco 406 S, sterilized by 15 min heating at 130°C, are added 2.5 ml of sterile 5% $NaHCO_3$; the pH is 7.4 in a 95% air–5% CO_2 atmosphere.

PBS (*xM* NaCl). The solution contains 0.01 M phosphate buffer, pH 7.2 (KH_2PO_4, Na_2HPO_4), with the specified NaCl concentration.

Dulbecco's PBS solution. It contains 10-fold diluted Gibco 408 and is sterilized by 15 min heating at 130°C; the pH is adjusted at 7.2 with sterile 5 N NaOH.

Saline–EDTA solution. The solution contains, per liter, 0.2 g EDTA, 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4; it is sterilized by 15 min heating at 130°C.

MEM solution. After dissolution in water of 10.7 g of minimum essential medium (Eagle) powder (with spinner salts and L-glutamine; Gibco F-14) and 2.2 g $NaHCO_3$, 4.0 ml of phenol red solution are added (0.05 g/l; Gibco 510); the solution is then brought to 1 liter with water and sterilized by passing through a 0.45-μm millipore filter.

MEM antibiotic solution. To 1 liter of MEM solution are added 5 ml of antibiotic solution (penicillin-streptomycin; Gibco 514). Sterilization is carried out by passing the solution through a 0.45-μm millipore filter.

Thymidine-saline solution. It contains 0.1 g of thymidine (Sigma) and 9g NaCl per liter of water.

B. *Cell lines*

Ehrlich cells. The cell line was received from Dr. V. Fredette, Armand Frappier Institute, Montreal. Male Swiss white mice are injected intraperitoneally with 10^7 cells in 0.2 ml of Dulbecco's PBS. The ascites fluid, collected 7–8 days later, is centrifuged 2 min at 500 × *g*. The cells are then washed twice with 10 ml of Dulbecco's PBS and finally resuspended in MEM solution (approximately 2.9×10^6 cells/ml).

HeLa cells. The cell line came from Dr. T. P.

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Abbreviations: TCA = trichloroacetic acid; EDTA = ethylenediaminetetraacetic acid; PPO = 2,5-diphenyloxazole; dimethyl-POPOP = 1,4-bis-2-(4-methyl-5-oxazolyl)-benzene; dTMP, dTDP and dTTP = thymidine monophosphate, thymidine diphosphate and thymidine triphosphate, respectively.

Brent, then at the McGill Cancer Research Unit, Montreal. The cells are grown as monolayers in MEM-antibiotic solution supplemented with 5% calf serum (Tissue Culture Service, Ltd.). After 1 week, they are detached with saline–EDTA solution, washed twice with 10 ml of Dulbecco's PBS, and resuspended in MEM solution (approximately 2.9×10^6 cells/ml).

C. *Inhibitor assays*

The inhibitor preparation (0.2 ml sample), or an equal volume of solvent (control), is mixed with 0.7 ml of MEM solution containing 2×10^6 cells (hemacytometer count).

In the direct assay [methyl-^{3}H]thymidine (4 μCi; 6 Ci/mmole; New England Nuclear) in 0.1 ml of Hanks' solution is immediately added bringing the total volume to 1 ml and the incubation carried out for 15 min at 37°C, with gentle shaking, in a 95% air–5% CO_2 atmosphere.

In the preincubation assay, the cells are kept for 2 hr at 37°C with the inhibitor preparation (or an equal volume of solvent) before addition of the labeled thymidine. The test is then pursued as described for the direct assay.

In both cases, the incubation is stopped by cooling the tubes in ice and adding 10 ml of cold thymidine–saline solution. After shaking on a Vortex mixer, the cell suspension is filtered on a fiber glass filter (Whatman GF/C); the filter is washed twice with thymidine saline solution, then with 10 ml of cold 10% trichloroacetic acid (TCA) and 10 ml of ethanol. The filter is transferred in a vial and dried before the addition of 10 ml of scintillating solution (4 g of PPO, 0.1 g of dimethyl-POPOP/liter of toluene). The radioactivity, counted in a Packard Tri-Carb spectrometer, after correction for the background, is given in counts/min. With Ehrlich cells and no inhibitor, the standard deviations on the radioactivity measurements are 6 and 22% in the conditions of the direct assay and preincubation assay, respectively.

The biological activity of the inhibitor preparation is estimated from the fractional decrease (I.F. = inhibited fraction) of labeled thymidine incorporation into the TCA-insoluble cell fraction:

$$\text{I.F.} = \frac{\text{"control"} - \text{"sample"}}{\text{"control"}}$$

where "sample" and "control" are the radioactivities of the TCA-insoluble fractions from the cells incubated with and without inhibitor respectively. A negative value of I.F. indicates a stimulation of the incorporation of labeled thymidine.

D. *Protein determination*

The protein is determined by the micromethod of Lowry *et al.* [4] with Sigma bovine serum albumin as standard.

EXPERIMENTS AND RESULTS

A. *Preparation and biological activity of supernatant from bovine mammary glands*

The mammary glands of a cow, taken immediately after the animal has been killed, are cut in large fragments which are cooled on ice. From then on, unless otherwise stated, all manipulations are carried out at 4°C. The mammary tissue is minced into small pieces with scissors, homogenized in water (2 ml/g of tissue) with a Waring Blendor for 90 sec and lyophilized; the powder is kept at −20°C. When needed, this powder is dispersed in PBS (0.15 M NaCl) (100 mg of powder/ml) with a Potter–Elvehjem homogenizer (25 strokes). The suspension is centrifuged at 10,000 rev/min for 10 min in the rotor SS-34 of a RC-2B Sorvall centrifuge. The recovered supernatant is then spun for 120 min at 30,000 rev/min in the rotor 30 of a Beckman L2-65 centrifuge; the clear zone, between the upper and lower turbid layers, is carefully taken with a Pasteur pipette (105,000 × *g* supernatant).

Ehrlich and HeLa cells are preincubated for various times, from 0 to 120 min, in the presence

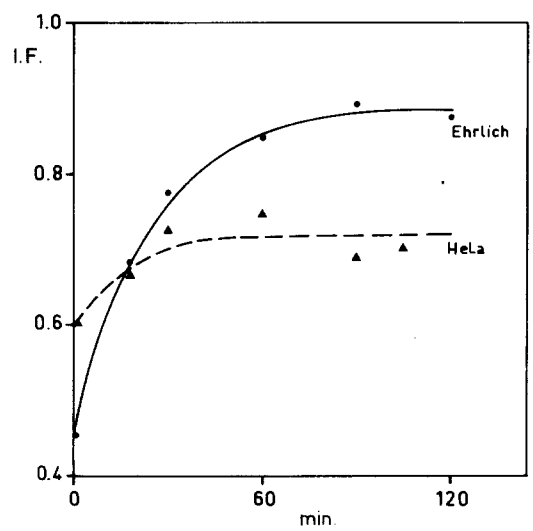

Fig. 1. Action of preincubation on the inhibition of labeled thymidine incorporation into DNA by the supernatant of bovine mammary glands. The cells are preincubated with the supernatant for the time indicated (min) *before addition of labeled thymidine; the DNA radioactivity is measured* 15 min *later. The decrease of radioactivity relative to a control without supernatant (I.F.) is calculated.* (●) *Ehrlich cells;* (▲) *HeLa cells.*

or absence of mammary 105,000 × *g* supernatant (3.2 mg of protein in 0.2 ml) before addition of the labeled thymidine and incubation in the conditions of the direct assay (see "Materials and Methods"). Figure 1 shows that, when there is no preincubation, the supernatant is more active on HeLa cells than on Ehrlich cells; but the inhibition of labeled thymidine incorporation into the DNA of Ehrlich cells increases with the preincubation time, and, when this preincubation time reaches 2 hr, the supernatant is more active on Ehrlich cells than on HeLa cells. We decided to purify the inhibitor which is more active on Ehrlich cells after a 2-hr preincubation.

7.5 ml are collected and the absorbance at 230 nm is read on each fraction.

To measure the inhibitory activity, the chromatography is run in dilute PBS; the fractions are pooled as indicated in Fig. 2. Aliquots (5 ml) are lyophilized and the residue redissolved in 0.5 ml of water; after centrifugation, 0.2-ml portions of the clear supernatant are used for the assays. Suitable controls are prepared with the chromatography solvent in order to calculate I.F. Two main peaks of inhibitory activity are separated (Fig. 2).

Because PBS (0.15 M NaCl) is used in the next purification step, the Sephadex G-50 chromatography is repeated in this solvent.

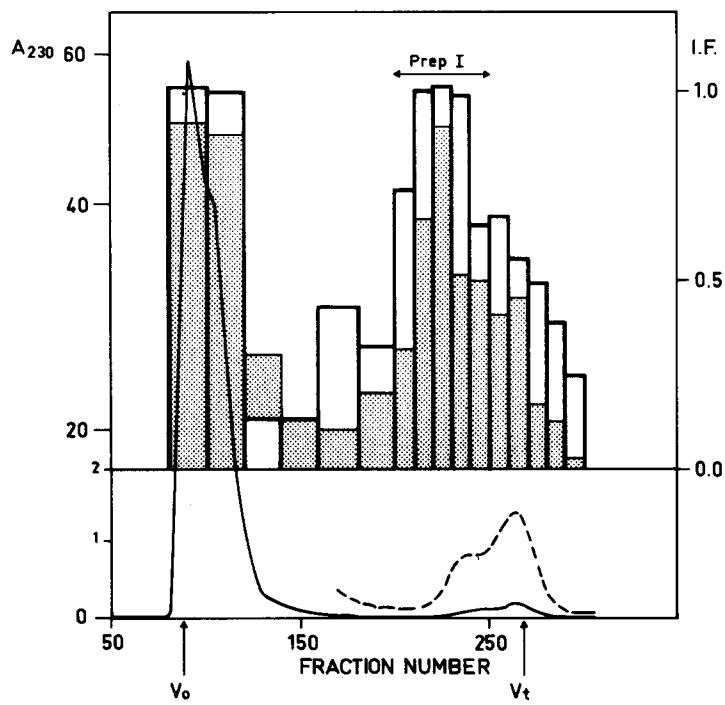

Fig. 2. Sephadex G-50 chromatography. The Sephadex G-50 chromatography is run in 10-fold diluted PBS (0.15 M NaCl). The absorbance at 230 nm (A_{230}) is read on each fraction; the continuous line corresponds to the 0–60 scale, whereas the dashed line corresponds to the 0–2 scale. The fractions are then pooled as indicated and aliquots used to determine the biological activity (see text); I.F. values are calculated relative to controls prepared with the chromatography solvent. Light frames surrounding stippled areas give the results of the direct assay, whereas the heavy frames give the results of the preincubation assay.

B. *Purification of the inhibitor*

Sephadex G-50. A column (5 × 100 cm; Pharmacia) is filled with Sephadex G-50 fine and equilibrated with PBS (0.15 M NaCl) or 10-fold diluted PBS (0.15 M NaCl) in the cold room. The clear 105,000 × *g* supernatant (100 ml; 1.6 g of protein) is applied to the column and the elution performed with PBS or dilute PBS at a flow rate of 100 ml/hr; fractions of

Determination of the biological activity is not possible because of the high salt concentration, but the absorbance curve at 230 nm is identical with that obtained with dilute PBS so that we can locate the two main peaks of inhibitor. Peak I is constituted of high molecular weight compounds; because some of them might be enzymes destroying the labeled thymidine or an amino-acid in the incubation medium, the lower molecular weight inhibitor (Peak II; K_{av}

$=0.91$)* is selected for further purification. Fractions 200 to 250 are pooled to give Prep I.

CM-Sephadex. A column (2.6 × 70 cm; Pharmacia) is filled with CM-Sephadex C-25 and equilibrated with PBS (0.15 M NaCl) in the cold room. Prep I from four Sephadex G-50 chromatographies run simultaneously in PBS (0.15 M NaCl) are pooled (1600 ml; 96 mg of protein) and applied to the CM-Sephadex column at a flow rate of 80 ml/hr; the elution is performed with PBS (0.70 M NaCl) at the same flow rate. Fractions of 7.5 ml are collected and the inhibitory activity is measured by the direct and preincubation assays.

Figure 3 shows that the rapid transition from 0.15 to 0.70 M NaCl concentration in PBS is associated with an increase of inhibitory activity in the fractions which is relatively more important for the direct assay. In spite of the fact that, from fraction 270 onward, most of the inhibition, measured against a control containing PBS (0.15 M NaCl), is likely due to the high osmolality, two peaks of inhibitors are detected by the preincubation assay: peak II, which is more important and associated with less protein, is chosen for further purification. Fractions 350 to 366 are pooled to form Prep II.

Concentration step. Prep II (112 ml) is diluted with phosphate buffer without NaCl (411 ml) to have a final 0.15 M NaCl concentration. This solution is applied, in the cold room, at a flow rate of 25 ml/hr, to a column (0.9 × 15 cm;

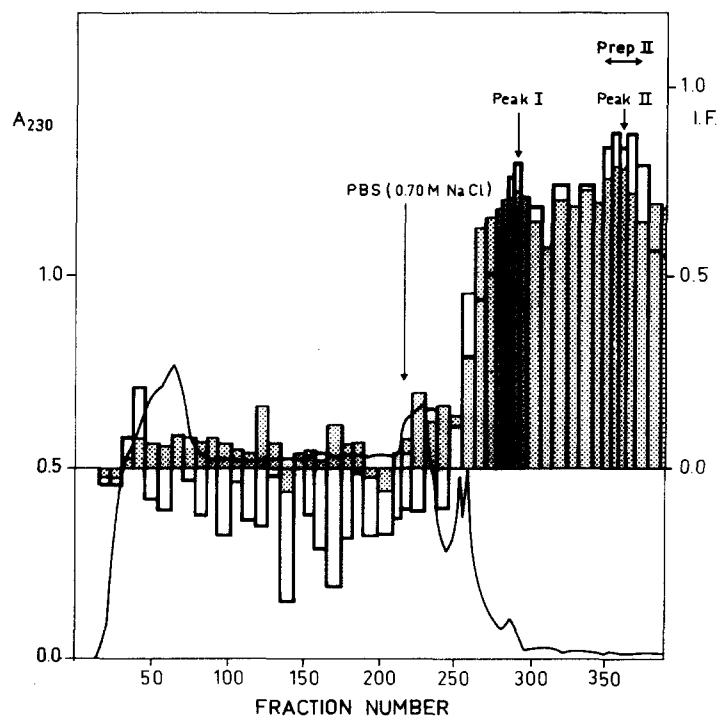

Fig. 3. CM-Sephadex chromatography. Prep I (*Fig.* 2) *in PBS* (0.15 M NaCl) *is applied to the CM-Sephadex column; elution with PBS* (0.70 M NaCl) *starts where indicated. The absorbance at* 230 nm (A_{230}) *is read on each fraction* (*continuous line*). *The fractions are then pooled as indicated and the biological activity is measured on* 0.2-ml *aliquots from each pool;* 0.2 ml *of PBS* (0.15 M NaCl) *is used for the control and I.F. calculated. Light frames surrounding stippled areas are used for the direct assay, whereas heavy frames are used for the preincubation assay.*

Pharmacia) filled with CM-Sephadex C-25 and previously equilibrated with PBS (0.15 M NaCl). The elution is performed with PBS (1.15 M NaCl) and 1-ml fractions are collected. Figure 4 shows that the inhibitor is concentrated in 5 ml of eluent (Prep III).

Sephadex G-25. Prep III (5 ml; 0.53 mg of protein) is applied, in the cold room, at a flow rate of 20 ml/hr, to a column (1.6 × 100 cm; Pharmacia) filled with Sephadex G-25 fine and

*Elution constant

$$K_{av}=\frac{V_e-V_o}{V_t-V_o}.$$

The void volume V_o is measured with blue dextran (Pharmacia) whereas the total volume V_t is calculated from the dimensions of the gel. V_e is the elution volume corresponding to the peak maximum.

previously equilibrated with PBS (0.15 M NaCl). The elution is carried out with the same buffer; fractions of 2.6 ml are collected. Figure 5 shows one main peak of inhibitory activity centered on a small absorption peak at 230 nm; the elution constant $K_{av}=0.53$ corresponds to that of a 2000–3000 mol. wt peptide. Fractions 50–56 are pooled to form Prep IV.

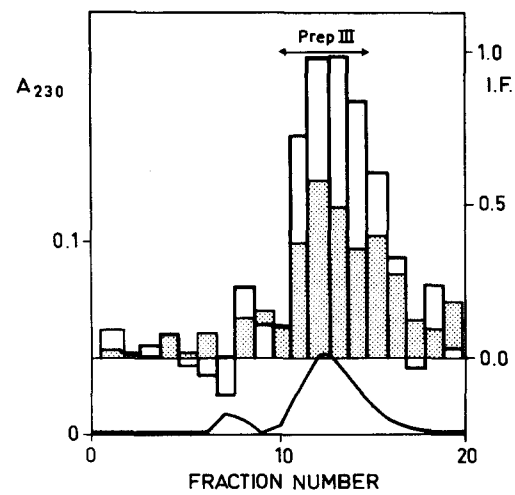

Fig. 4. Concentration on CM-Sephadex. Prep II (*Fig.* 3), *diluted in such a way that the solvent becomes PBS* (0.15 M NaCl), *is applied onto a short CM-Sephadex column; elution with PBS* (1.15 M NaCl) *and collection of the fractions start together. The absorbance is read at* 230 nm (A_{230}; *continuous line*). *Aliquots of* 20 µl *from each fraction are used for the biological assays; suitable controls enable us to calculate I.F. Light frames surrounding stippled areas are used for the direct assay, whereas heavy frames correspond to the preincubation assay.*

C. *The inhibitor unit; calculation of yield and purification factor*

Increasing amounts of Prep IV in PBS (0.15 M NaCl) are tested in the conditions of the direct and preincubation assays (Fig. 6). The unit is defined as the amount of the inhibitor needed to have an I.F. of 0.50 in the conditions of the preincubation assay. The aliquot introduced in the assay medium is diluted so that I.F. is between 0.50 and 0.70 and the number of inhibitor units is calculated from the curves of Fig. 6 corresponding to the preincubation assay.

Table 1 gives the number of units and the specific activity (units per mg of protein) at each step of purification. An overall yield of 12.5% and a final purification factor of 2300 are calculated; these are minimum values as shall be discussed later.

D. *Polyacrylamide gel electrophoresis*

The electrophoreses are carried out in a cooled bath, using β-alanine–acetic acid, pH 4.5 buffer, at 3 mA per tube, for 120 min with the native

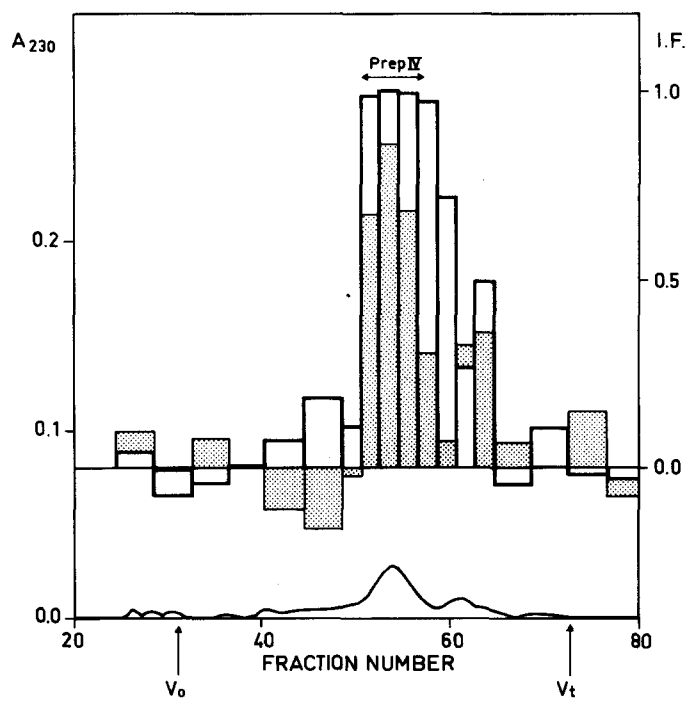

Fig. 5. Sephadex G-25 chromatography. Prep III (*Fig.* 4) *is applied to a Sephadex G-25 column which is eluted with PBS* (0.15 M NaCl). *The absorbance at* 230 nm (A_{230}) *is read on each fraction* (*continuous line*). *The fractions are then pooled as indicated and* 0.2-ml *aliquots are taken for the biological assays; suitable controls are prepared to calculate I.F. Light frames surrounding stippled areas give the results of the direct assay, whereas heavy frames are used for the preincubation assay.*

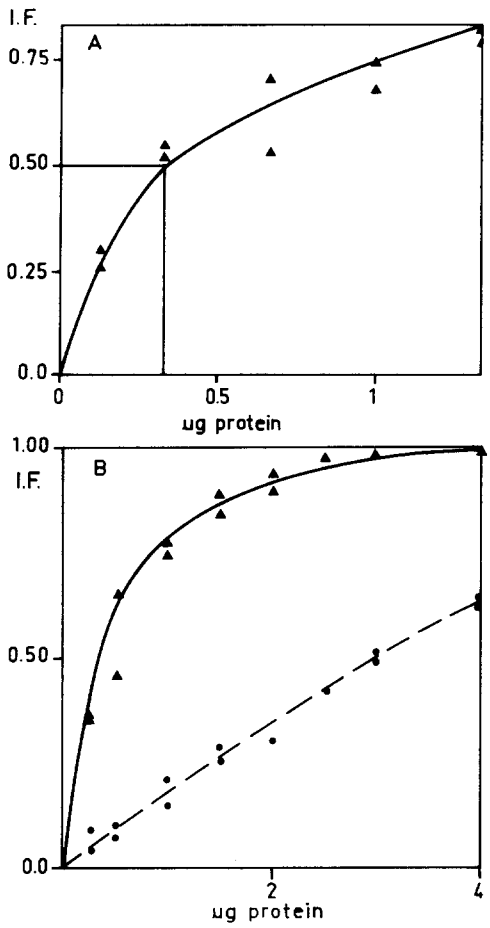

Fig. 6. Dose–effect relationship with the purified inhibitor (Prep IV). The fractional decrease (I.F.) of labeled thymidine incorporation into Ehrlich cell DNA is given as a function of the amount (μg protein) of Prep IV (Fig. 5). (●) direct assay (part B; dashed line); (▲) preincubation assay (parts A and B; continuous line).

inhibitor and 150 min with the inhibitor in 8 M urea. The gels stained with Coomassie blue are scanned at 550 nm.

Prep IV is desalted by passing through a Sephadex G-25 column run in water or in 0.05 mM EDTA.

Gel electrophoresis of native inhibitor. A slightly modified standard method is used [5]. The gel, which contains 6% acrylamide and 0.32% bisacrylamide (w/v), is submitted to a preelectrophoresis for 4 hr in potassium acetate, pH 4.3 buffer. The solution of the inhibitor in 0.05 mM EDTA is lyophilized, the residue is dissolved in 10% sucrose and 15-μl aliquots (20 μg of protein) are applied immediately on two identical gels. After electrophoresis, one gel is stained with Coomassie blue; the densitometric record presented in Fig. 7 shows a single absorption band. The other gel is cut into 4-mm slices and each slice is crushed with a glass rod in 0.6 ml of

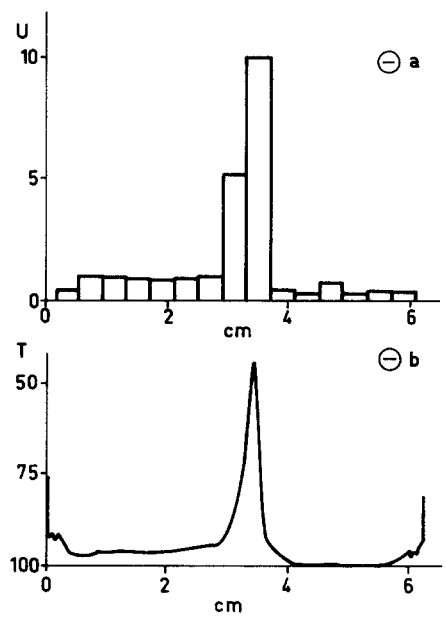

Fig. 7. Gel electrophoresis of the native inhibitor. Aliquots of desalted Prep IV are applied on two identical gels and the electrophoresis is carried out in the conditions described in the text. One gel is stained with Coomassie blue, scanned at 550 nm and the percent transmittance (T) is recorded as a function of the migrated distance (cm) toward the cathode (part b); the other gel is cut in 4-mm slices for the determination of the biological activity (U = number of inhibitor units) (part a).

Table 1. Purification of the inhibitor

Purification step	Units	Yield %	Protein mg	Specific activity units/mg	Purification factor
Supernatant	4900	100	6150	0.8	1
Prep I	800	16	97	8.3	10
Prep II					
Prep III	725	14	0.53	1360	1700
Prep IV	611	13	0.33	1850	2300

After determination of I.F. in the preincubation assay, the number of inhibitor units is calculated; the specific activity is the number of units per mg of protein. Yields and purification factors are calculated relative to the values found in the supernatant. Prep I is obtained from Sephadex G-50 (Fig. 2), Prep II from CM-Sephadex (Fig. 3), Prep III from the concentration step (Fig. 4) and Prep IV from Sephadex G-25 (Fig. 5). The high salt concentration in the fractions from the CM-Sephadex (Prep II) did not allow a reliable estimation of the biological activity.

0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 buffer; after centrifugation, the biological activity is measured on 100-μl aliquots from the supernatant and the number of inhibitor units is calculated. Figure 7 shows that the biological activity migrates as a single peak which has the same position in the gel as the band colored with Coomassie blue.

Gel electrophoresis in 8 M *urea.* The solution of the inhibitor in water is lyophilized, the residue is dissolved in 40% sucrose, 8 M urea and 15-μl aliquots (20 μg of protein) is applied to the gel. The method of Traub *et al.* [6] is followed. The stacking gel contains 2.5% acrylamide and 0.625% bisacrylamide, whereas the separating gel contains 10% acrylamide and 0.2% bisacrylamide (w/v). The densitometric record of the gel stained with Coomassie blue shows a single absorption band (Fig. 8).

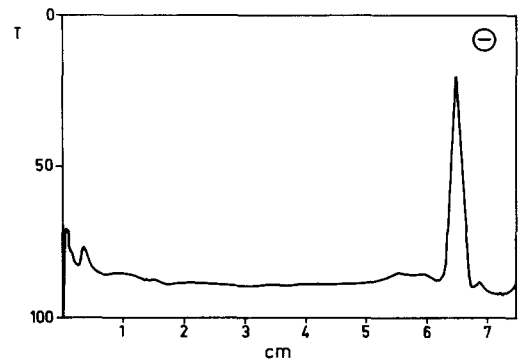

Fig. 8. Gel electrophoresis of the purified inhibitor in 8 M *urea. An aliquot of desalted Prep* IV *is submitted to gel electrophoresis in* 8 M *urea as described in the text. The gel is colored with Coomassie blue and scanned at* 550 nm. *The figure gives the percent transmittance* (*T*) *as a function of the migrated distance* (cm) *toward the cathode.*

E. *Some properties of the purified inhibitor*

Action of preincubation time on the inhibition of [^{3}H] *thymidine incorporation into Ehrlich cell DNA by Prep* IV. Ehrlich cells are preincubated for 0–120 min in absence [control with PBS (0.15 M NaCl)] and in presence of the purified inhibitor [Prep IV (2 and 12 units) in the same buffer], the [^{3}H] thymidine is added and the incubation is pursued under the conditions of the direct assay. The results are given in Fig. 9. For the smaller dose (2 units), there is, during the first 30 min, a fast increase of I.F. which remains then unchanged for longer times. For the higher dose (12 units), I.F. increases rapidly during the first 15 min, then at a slower and steady rate.

Effect of incubation at 37°C *on the inhibitor biological activity.* The inhibitor [Prep IV in PBS (0.15 M NaCl); 20 μg of protein/ml] and a control (buffer alone) are incubated at 37°C for 0–300 min before measuring the biological activity on Ehrlich cells under the conditions of the direct and preincubation assays. Figure 10 shows that the inhibitory activity tested in the direct assay increases during the first 30 min, then decreases; after an incubation of 4 hr, the biological activity has practically disappeared from the preparation. When tested in the preincubation assay (the preparation is first incubated for the indicated time, the cells are then added and the suspension is preincubated for a further 2 hr), there is no initial activation and, after a plateau of 60 min, the inhibitory activity decreases to be negligible after 4 hr.

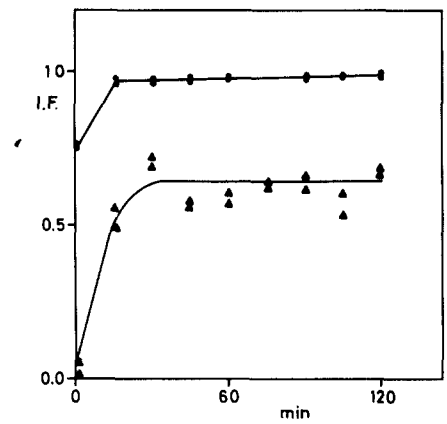

Fig. 9. Action of preincubation on the inhibition of labeled thymidine incorporation into DNA by Prep IV. *The cells are preincubated with Prep* IV *for the time indicated* (min) *before addition of labeled thymidine; the DNA radioactivity is measured* 15 min *later. The decrease of radioactivity relative, to a control without Prep* IV (*I.F.*) *is calculated.* (●) 12 *units;* (▲) 2 *units.*

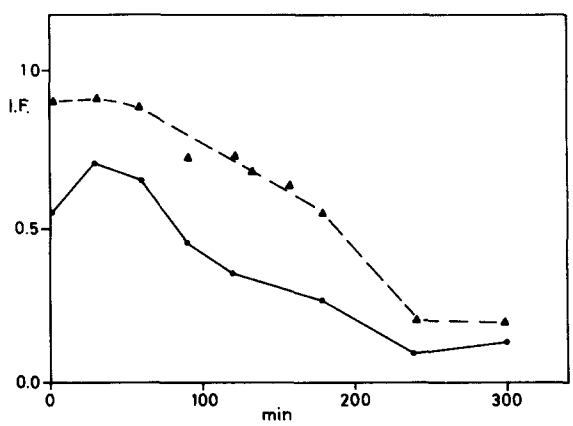

Fig. 10. Action of incubation at 37°C *on the biological activity of Prep* IV. *Prep* IV *is incubated at* 37°C *for the time indicated* (min) *before addition of cells; the biological activity is then measured by the direct assay* (●) *or the preincubation assay* (▲).

DISCUSSION

If the action is tested immediately, the supernatant of bovine mammary glands inhibits the exogenous thymidine incorporation into DNA more in HeLa cells (maligant cells derived

from the cervix epithelium of human uterus) than in Ehrlich cells (malignant cells from mouse mammary gland). On the contrary, the action is greater on Ehrlich cells than on HeLa cells when the cells are preincubated for 2 hr with the supernatant before addition of the thymidine. The supernatant thus seems to contain an inhibitor rather specific for Ehrlich cells the action of which on thymidine incorporation into DNA increases during the first 2 hr of contact with the cells. This inhibitor has been purified in three chromatographic steps: Sephadex G-50, CM-Sephadex, and Sephadex G-25 (the second CM-Sephadex before the Sephadex G-25 is only a concentration step). The elution constant from the Sephadex G-25 ($K_{av}=0.53$) indicates a rather small molecular weight. Gel electrophoresis at pH 4.3 gives a single band stained by Coomassie blue corresponding exactly to the inhibitory activity; the purity of the final product is confirmed by gel electrophoresis in the presence of 8 M urea which also gives a single band after staining with Coomassie blue.

We have defined an inhibitor unit and studied, with the purified inhibitor, the relationship between dose and effect on labeled thymidine incorporation into Ehrlich cell DNA. This enables us to calculate the total number of units and the specific activity (number of units per mg of protein) at each step of purification (Table 1). A 13% overall yield and a purification factor of 2300 are calculated for the purified inhibitor; the true values must be higher because reference is made to the 105,000 × *g* supernatant which contains many other inhibitors.

Inhibition of exogenous [^{3}H]thymidine incorporation into DNA does not mean necessarily a decrease in DNA synthesis: the same results can be obtained by dilution of the labeled precursor with nonradioactive thymidine [7, 8], by addition of enzymes destroying thymidine [9], by inhibition of thymidine kinase, or by a greater contribution of the endogenous synthesis of the DNA precursors. We have reported elsewhere [3] that the purified mammary inhibitor truly inhibits DNA synthesis: in Ehrlich cells incubated with [^{3}H]thymidine, Prep IV increased the radioactivity in the three phosphorylated derivatives of thymidine (dTMP, dTDP and dTTP) while producing a strong decrease of the DNA radioactivity; the metabolic block is thus between dTTP and DNA. The incubation medium also contained [^{14}C]orotate; ^{14}C incorporation into DNA was inhibited to approximately the same extent as the ^{3}H incorporation showing that the endogenous synthetic pathway and the salvage pathway are both affected; ^{14}C incorporation into RNA was the same as in the control which suggests that the inhibitor does not disturb the overall RNA metabolism. We also showed [3] that the purified mammary inhibitor is far more active on the three kinds of mammary cells that were tested (Ehrlich, Walker and isolated mammary cells from mice at mid-gestation) than on cells deriving from other tissues (chick embryo, Novikoff and HeLa cells).

The 2-hr preincubation, before addition of [^{3}H]thymidine, used in the assay to follow the inhibitor specific for mammary cells was chosen after an experiment with the crude mammary supernatant which contains many nonspecific factors (Fig. 1). The influence of preincubation had thus to be reinvestigated with the purified inhibitor (Fig. 9); for the smaller dose used (2 units), which is about the amount used to quantitate the inhibitor, I.F. reaches after 30 min a plateau which still lasts at 120 min. It thus appears that adequate conditions were chosen to measure the number of units at each step of purification.

The purified factor is quite labile at 37°C (Fig. 10), being practically inactivated after 2 hr. Drastic conditions, like lyophilization, destroy the activity. Therefore the purification is carried out in a continuous way without lyophilizing or freezing the intermediate preparations. After the final step, the purified inhibitor (Prep IV) can be kept frozen at −20°C in PBS (0.15 M NaCl) with little loss of activity.

Tissue growth appears to be controlled by two kinds of factors: stimulatory and inhibitory. The inhibitory factors might be produced by the tissues themselves so that their growth would be regulated by a negative feedback mechanism. Inhibitors of this kind were named "chalones" by Bullough [10]; chalones are not species-specific, but they are tissue-specific: the action of a chalone is limited to the tissue which releases it. We have purified from cow mammary glands a DNA synthesis inhibitor of small molecular weight which appears specific for mammary cells, but which is devoid of species-specificity; the bovine inhibitor is active on murine mammary cells. It might be a mammary chalone. The isolated compound is interesting from a fundamental point of view as a tool to probe the control mechanism of the cellular division cycle; because of its activity on malignant cells derived from breast tumors, it might also be interesting from a clinical point of view considering the high incidence of mammary carcinoma.

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Immunosuppression in Mice Bearing Primary Tumors*

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Abstract—*Spleen cells from C57Bl/6, BALB/c and CBA/J mice bearing primary methylcholanthrene induced sarcomas and C3H/HeJ mice bearing primary spontaneous mammary tumors gave suppressed responses to mitogens. Those spleens also contained a population of suppressor cells which inhibited the mitogen responses of normal spleen cells. Immunoglobulin rich fractions of sera from all these mice except the BALB/c tumor bearers, contained a suppressive factor which inhibited the mitogen response of normal syngeneic spleen cells.*

INTRODUCTION

MOST studies in tumor immunology have been performed using transplanted tumors in animal systems. There is little doubt that a considerable amount of valuable information has been obtained from such studies concerning the relationship of tumor growth to the immune system. However, it seems necessary to demonstrate that the observed effects in transplanted tumor systems correspond to the effects of primary tumors on the immune system, for such studies would more closely correspond to the human situation.

In previous work from this laboratory, using a variety of transplanted tumors, suppression of general immunity [1] and development of suppressive serum factors [2, 3] and suppressor cells [4] in mice with progressively growing tumors has been reported. Others have reported similar observations in both animals [5–8] and humans [9–12]. Thus, much evidence now supports the contention that progressive tumor growth results in progressive immunosuppression and this immunosuppression may be due to the development of suppressor cells and/or suppressive serum factors.

The purpose of the present experiments was to test immune competence and suppression in mice of different strains bearing chemically-induced primary sarcomas or spontaneous mammary tumors. The similarity of the results from these mice with previous results from mice bearing transplantable tumors serves to strengthen the concepts and to show a closer correlation with the human situation. Although these studies are somewhat limited in their scope, in that only a limited number of assays could be done on each animal bearing a primary tumor, all the observations indicate a close similarity between primary and transplantable tumors, in their effect on general immune competence.

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MATERIALS AND METHODS

Mice

BALB/c, CBA/J and C57Bl/6J females aged 6–8 weeks were obtained from Jackson Laboratory and were used when 2–4 months old. Retired C3H/HeJ breeders were also obtained and used as a source of spontaneous mammary tumors. Age matched controls were maintained and used throughout.

Tumors

Primary sarcomas were induced in BALB/c, CBA/J and C57/Bl mice by intramuscular injection in the right hind leg of 0.5 mg of 3-methylcholanthrene (MCA) suspended in 0.1 ml trioctanoin. Spontaneous mammary tumors developed in a high proportion of the C3H/HeJ females at an age of about 12 months.

Collection and preparation of serum

Blood was collected from the retro-orbital sinus or by exsanguination from the heart of normal or tumor-bearing mice (tumors > 3.0 g). The blood was allowed to clot overnight at 4°C after which the sera were separated, inactivated at 56°C for 30 min and stored at −20°C.

Fractionation and assay of mouse serum

Whole serum was fractionated on Sephadex G-150 in order to obtain the immunoglobulin containing fraction. The Ig peak was precipitated with 40% saturated $(NH_4)_2SO_4$ and reconstituted to its original volume in physiological saline as previously described [13]. The immunosuppressive activity of the Ig fraction was assessed by titration at various concentrations in culture with ConA and normal spleen cells from the strain syngeneic to the tumor bearer.

Culture system for mitogen stimulation

These methods have been described in detail previously [1–4]. Spleen cells were cultured in 0.2 ml of RPMI-1640 medium containing 50 μg/ml gentamycin in microtiter plates with 5% fetal calf serum (FCS) in tests for competence and suppression by tumor bearer spleen cells and 2% FCS in tests with serum preparations. Mitogens were added in 0.05 ml vol at final concentrations of 4 μg/ml for concanavalin A (ConA) or 25 μg/ml for lipopolysaccharide (LPS). One μCi of ^{3}H-thymidine was added to each culture after 48 hr and the cultures were harvested 18 hr later.

Enrichment of suppressor cells by centrifugation on Hypaque–Ficoll

This method was completely described elsewhere [4].

Data presentation

Mitogen stimulation results were expressed as mean CPM of 3 replicate cultures. Standard deviations were always less than 10%. Differences greater than 15% are statistically significant by Student's *t*-test at the level of $P = < 0.05$.

RESULTS

Mice developing primary tumors were sacrificed and heart bled at various times after tumor development. General lymphocyte competence in these primary tumor-bearers was assessed by their spleen cell responses to ConA, a T cell mitogen [14] and LPS, a B cell mitogen [15]. Significantly depressed mitogen responses were observed in all animals with tumors >3.0 g and in some animals with tumors between 2.0 and 3.0 g. This was the case in all the species tested regardless of whether the tumor was a primary MCA-induced sarcoma or a spontaneous mammary tumor. Results of these studies are shown in Tables 1–3. It can be seen that with the smaller tumors (<3.0 g) responsiveness to ConA was either normal or close to the normal range. However, as tumors progressed there appeared to be a loss of competence which correlated roughly with tumor size. The LPS response in the various mouse strains appeared, in general, to be less affected by the presence of tumor, although suppression of the response was noted in mice with large tumors. The exception to this rule was the C3H/He mice with mammary tumors. Normal mice of this strain had very low responses to LPS, and they did not appear to be markedly altered during tumor growth.

Spleen cells from primary tumor-bearers

Table 1. *General immune competence of normal and tumor-bearer spleen cells assessed by responsiveness to mitogens*

Strain	Spleen source*	Tumor weight (g)	^{3}H-thymidine incorporation (CPM)†		
			None	Con A	LPS
C57/Bl6	N_1		5360	209,000	78,200
	N_2		10,300	153,000	86,200
	T_1	2.64	6060	142,000	54,500
	T_2	5.70	4240	2140	39,400
	T_3	6.22	3620	9920	11,300
C3H/He	N_1		9380	325,000	32,700
	N_2		10,800	203,000	15,100
	T_1	2.37	19,200	165,000	26,600
	T_2	2.66	20,100	286,000	45,400
	T_3	2.75	25,800	83,300	30,300
	T_4	2.99	18,300	31,400	30,300
	T_5	4.54	13,100	31,800	18,500
	T_6	5.47	9510	1800	10,400
	T_7	5.57	14,100	5060	25,600

*8×10^5 spleen cells from normal (N) or tumor bearer (T) mice were tested.
†The mean CPM of triplicate cultures are given.

Table 2. General immune competence of normal and tumor-bearer spleen cells from CBA/J mice assessed by responsiveness to mitogen

Spleen source*	Tumor weight (g)	^{3}H-thymidine incorporation (CPM)†		
		None	ConA	LPS
N_1		16,900	172,000	58,500
N_2		21,519	263,000	137,900
N_3		36,900	233,000	103,000
N_4		18,700	250,000	150,000
N_5		7080	175,000	89,700
T_1	2.3	5670	103,000	50,300
T_2	2.7	5330	218,000	46,200
T_3	3.7	7490	24,400	19,500
T_4	5.25	12,800	75,600	17,500
T_5	5.75	15,400	920	3280
T_6	6.8	16,700	8960	51,600
T_7	9.2	6430	720	6590
T_8	9.42	6740	710	6820

*8×10^5 spleen cells from normal (N) or tumor-bearer (T) mice were tested.
†The mean CPM of triplicate cultures are given.

Table 3. General immune competence of normal and tumor-bearer spleen cells from BALB/c mice assessed by responsiveness to mitogen

Spleen source*	Tumor weight (g)	^{3}H-thymidine incorporation (CPM)†		
		None	ConA	LPS
N_1		32,800	227,300	87,600
N_2		32,500	220,000	91,900
N_3		13,100	218,000	93,500
N_4		19,600	187,000	57,800
N_5		9450	214,000	80,400
T_1	2.00	12,000	168,000	65,200
T_2	2.70	13,700	138,000	41,400
T_3	4.20	15,800	110,000	28,900
T_4	6.30	20,700	106,000	43,200
T_5	6.4	6200	23,200	29,000
T_6	7.9	9060	53,900	22,400
T_7	9.2	8620	1430	17,800

*8×10^5 spleen cells from normal (N) or tumor bearer (T) mice were tested.
†The mean CPM of triplicate cultures are given.

were tested for their ability to suppress the mitogen responses of normal spleen cells [4]. Spleen cells to be tested were taken from mice with large tumors (>3.0 g) for this experiment. The results are shown in Table 4. Spleen cells from every animal bearing a primary tumor significantly suppressed the ConA response of normal spleen cells with the exception of CBA/J (T_3). Centrifugation of tumor bearer spleen cells on Hypaque–Ficoll produced an enriched population of suppressor cells in the light fraction [4] from all mice but two C3H tumor bearers (T_1, T_2). It appears that suppressor activity may be enriched for by cell separation, for spleen cells from CBA/J (T_3) were not suppressed as a whole population. However, when they were separated on Hypaque–Ficoll, they did show a degree of suppression. The LPS responses were also suppressed to some extent by the spleen cells of the primary tumor bearers, but to a lesser extent than the ConA response. Cell separation did not appear to increase this suppressive effect. It has been shown previously in this laboratory, that separation of spleen cells from normal animals do not enhance for the suppressive effect with either mitogen.

In order to determine that the suppressor cell activity noted with tumor bearer spleen cells was not a unique aspect of the mitogen dose or the timing of the cultures, dose and timing studies were done with suppressor cells

Table 4. Effect of tumor-bearer spleen cells on mitogen stimulation of normal spleen cells

	Spleen cell combination*		^{3}H-thymidine incorporation (CPM)†		
	5×10^5	3×10^5	None	Con A	LPS
C57/Bl6J	N	N	5360	209,000	78,200
	N	T_1	6060	142,000	54,500
	N	T_1L	7040	740	39,200
	N	T_2	5220	20,100	59,200
	N	T_2L	9340	687	21,300
CBA/J	N	N	16,900	172,000	58,500
	N	T_1	20,700	6130	45,000
	N	T_1L	21,400	1400	32,500
	N	T_2	11,500	100,000	26,000
	N	T_2L	13,500	89,200	29,600
	N	T_3	16,400	178,000	42,900
	N	T_3L	14,000	131,000	44,800
BALB/c	N	N	19,600	187,000	57,800
	N	T_1	18,800	123,000	37,900
	N	T_1L	20,000	11,600	16,700
	N	T_2	20,400	129,000	34,000
	N	T_2L	19,600	28,100	18,400
	N	T_3	15,800	110,000	28,900
	N	T_3L	19,500	97,500	27,400
C3H/HeJ	N	N	9380	325,000	32,700
	N	T_1	15,200	183,000	34,000
	N	T_1L	22,300	204,000	36,700
	N	T_2	15,600	28,900	26,200
	N	T_2L	20,600	36,500	35,400
	N	T_3	17,000	77,600	27,400
	N	T_3L	17,800	29,300	32,000

*Cultures consisted of 5×10^5 normal spleen (N) plus 3×10^5 normal or tumor-bearer whole spleen (T) or the light fraction from Hypaque–Ficoll centrifuged tumor-bearer spleen (T_L).
†Results were given as mean CPM of triplicate cultures.

for the ConA response. Representative results are shown in Table 5. It can be seen that suppression was observable at all times and over a wide range of ConA doses. However, most consistent results were observable at 72 hr over the range of 1–8 μg/ml of ConA. This merely reflects the optimal timing and dose range in the unsuppressed cells.

The Ig rich fractions (Sephadex G-150) of serum from all C57/Bl, CBA/J and C3H/HeJ primary tumor bearers were highly immunosuppressive when compared to equivalent normal serum fractions (Table 6). In contrast, the BALB/c tumor bearers had no suppressive activity in their Ig fractions. The suppression caused by the serum of the tumor bearing animals was found to be independent of mitogen dose. A titration of a representative serum sample is shown in Table 7.

DISCUSSION

Previous studies from our and other laboratories have demonstrated that spleen cells from mice with progressively growing tumors usually exhibit depressed mitogen responses [1, 5, 6] indicating a general suppression of the ability of their lymphocytes to be stimulated. Further, these animals develop a population of cells in their spleens and a factor in their serum both of which can suppress mitogen responses of normal lymphoid cells [2–4, 8]. The nature and mechanism of action of the suppressor cells and factors are currently under extensive investigation by us and others with the aim of understanding the mechanism(s) of general immunosuppression in tumor-bearers. These studies seem to be of real importance considering that general immunosuppression is often observed in human cancer patients [9–12].

In view of the potential importance of this subject, it is essential to establish valid animal systems which will correlate with the human situation. All previous work with animals has involved the uses of transplanted tumors. Such tumors are very useful and have many advantages in that extensive studies can be done on large groups of tumor bearing animals. However, it seems necessary to show that these transplanted tumors behave similarly

Table 5. Dose and time response in assessing the suppressive activity of tumor-bearer spleen cells

Con A added (μg/ml)	CPM per culture* 5×10^5 N	5×10^5 N + 3×10^5 NL	5×10^5 N + 3×10^5 TL	% Suppression
		48 hr cultures		
None	11,000	20,000	18,000	
0.5	52,000	78,000	38,000	27
1	83,000	113,000	41,000	51
2	99,000	138,000	60,000	40
4	112,000	146,000	29,000	74
8	72,000	111,000	27,000	63
16	20,000	46,000	23,000	—
		72 hr cultures		
None	8,000	13,000	13,000	—
0.5	64,000	68,000	23,000	64
1	93,000	94,000	14,000	85
2	110,000	111,000	16,000	85
4	151,000	158,000	10,000	96
8	132,000	183,000	2,000	98
16	16,000	40,000	4,000	75
		96 hr cultures		
None	7,000	10,000	16,000	—
0.5	34,000	25,000	8,000	76
1	43,000	26,000	7,000	84
2	36,000	38,000	25,000	31
4	52,000	57,000	32,000	38
8	83,000	92,000	9,000	89
16	12,000	45,000	10,000	16

*Cultures consisted of 5×10^5 normal spleen (N), 5×10^5 normal spleen plus 3×10^5 normal cells from the light fraction from Hypaque–Ficoll separated spleen cells ($N + N_L$), or 5×10^5 normal splenocytes plus 3×10^5 light cells from tumor bearer spleen ($N + T_L$).

Table 6. Effect of the immunoglobulin fraction from normal mice and individuals bearing primary tumors on the uptake of ^{3}H-thymidine in Con A stimulated cultures of normal syngeneic mouse spleen cells

Mouse strain	Source of immunoglobulin fraction*	Mg protein added per culture	^{3}H-thymidine incorporation ConA†	% inhibition
C57Bl/6J	Normal‡	0.075	64,573	
	T_1	0.075	13,580	79
	T_2	0.075	31,916	51
	T_3	0.075	20,546	68
CBA/J	Normal	0.075	259,040	
	T_1	0.075	93,496	64
	T_2	0.075	46,890	72
	T_3	0.075	92,206	64
BALB/c	Normal	0.06	79,390	
	T_1	0.06	99,673	0
	T_2	0.06	109,540	0
	T_3	0.06	119,666	0
C3H/HeJ	Normal	0.06	113,000	
	T_1	0.06	56,800	50
	T_2	0.06	65,500	42
	T_3	0.06	57,900	49

*Serum was taken from normal or tumor bearing animals and fractionated on Sephadex G-150.
†Results are expressed as mean CPM of triplicate cultures followed by percent inhibition.
‡Normal serum samples comprised pooled serum from 3 to 6 mice.

*Table 7. Titration of ConA responsiveness in the presence of normal or tumor-bearer mouse serum**

ConA added μg/ml	5% FCS	CPM† from cultures containing normal mouse serum	tumour bearer serum	% inhibition
0	6000	4000	2000	—
0.25	1000	1000	1000	—
0.50	86,000	19,000	6000	68
1	109,000	74,000	26,000	65
2	155,000	143,000	50,000	65
4	226,000	173,000	75,000	57
8	150,000	220,000	76,000	65
16	—	126,000	2000	99

*Normal and tumor-bearer serum were tested at 1% of culture volume.
†The mean CPM of triplicate cultures are given.

to primary tumors which are more analogous to the human situation.

The data presented in this paper show that in general, mice of various strains with primary tumors have depressed mitogen responses indicating a probable suppression of general immune competence. While this assessment of immune status is indirect, it should be emphasized that in all previous work using transplantable tumor models, the correlation between lowered mitogen responsiveness and loss of specific immune functions was invariant and proportional [1]. We have also shown that animals bearing primary tumors have cells in their spleens which are capable of suppressing the mitogen responses of normal syngeneic spleen cells. These cells are enriched in the light fraction of Hypaque–Ficoll separated cells. These observations correlate directly with a study done in this laboratory on non-specific suppressor cells in mice bearing a transplantable methylcholanthrene induced tumor [4].

The Ig-rich fraction of the serum from mice bearing primary tumors was found to be immunosuppressive, as assessed by its ability to suppress mitogen responsiveness in normal syngeneic splenocytes. This also correlates directly with previous work carried out in this laboratory. This factor, found in the serum of mice bearing transplantable tumors, has been shown to inhibit not only mitogen responsiveness but also most specific *in vitro* immunologic functions [2, 3, 13]. Studies with immunoabsorbent columns have shown that the suppressive material may be some kind of immunoglobulin, since it can be removed by anti-mouse k and anti-mouse λ antisera [16]. We have no direct or conclusive evidence that the suppressive serum factor demonstrated in mice bearing primary tumors is identical to the factor characterized in mice with transplantable tumors. The paucity of serum obtainable from a single mouse with a primary tumor precludes such a study. However, insofar as was possible to test, it appeared that these serum factors were comparable.

An interesting observation was made that all BALB/c mice tested here had suppressed mitogen responses and splenic suppressor cells but no demonstrable suppressive serum activity. This was observed by us earlier with another primary spontaneous BALB/c tumor of unknown origin and also with a long established transplantable BALB/c melanoma [2]. The lack of suppressive activity is obviously not due to the tumor type as was originally thought but is instead a peculiarity of the BALB/c strain. Further work would determine whether this factor is indeed absent or is masked in some way which our separation procedures are not sufficient to unmask. In any event, it is not an unreasonable observation since there are numerous human cancer patients who do not exhibit serum inhibitors, despite their being immunosuppressed, even though other patients with identical tumor types do have suppressive factors. The present data provide further support for evidence of immunosuppression in individuals bearing progressively growing tumors.

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Potentiating Action of Cobra Venom Cytotoxin on the Antitumour Effects of an Alkylating Agent (Melphalan)

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Abstract—*Cytotoxin P_6 isolated from cobra venom which can inhibit the growth of several tissue culture cell lines and also the growth of Yoshida sarcoma in rats has been shown to considerably enhance, the growth inhibition produced by melphalan on HeLa and KB cells. It can also potentiate the effects of melphalan, on the growth of Yoshida sarcoma cells in rats. The effective dose of mephalan required to produce* 100% *inhibition of cell growth in rats bearing Yoshida sarcoma tumour could be decreased from* 0.65 μmole/kg *body weight to* 0.07 μmole/kg *body weight in animals which had been injected with cytotoxin P_6 four hours before receiving melphalan injection. In view of the earlier evidence showing that cytotoxin P_6 attacks the plasma membrane of susceptible cells, the potentiating effects described are presumably brought about by promoting preferential penetration of the nitrogen mustard into the tumour cells.*

INTRODUCTION

PREVIOUS studies [1] have demonstrated that a basic polypeptide (cytotoxin P_6) isolated from cobra venom shows preferential cytotoxicity to certain cells. The Yoshida sarcoma cells in particular have been found to be highly susceptible to destruction by the cytotoxin which is also able to prevent the growth of this tumour in rats. Other investigations have demonstrated that it binds with components on the cell surface of susceptible cells [2]. This paper describes experiments designed to examine the action of cytotoxin P_6 on the antitumour effects of the alkylating agent such as melphalan. It is consistent with the data obtained using tumour cell lines and rats bearing Yoshida sarcoma that the cytotoxin potentiates the antitumour effects of melphalan possibly by facilitating preferential penetration of the nitrogen mustard into tumour cells.

MATERIALS AND METHODS

Melphalan was kindly supplied by Dr. T. A. Connors, Chester Beatty Research Institute, London. Eagle's medium as modified by Dulbecco was obtained from BIOS, Bombay, India. Cytotoxin P_6 was prepared according to the procedure described earlier [1].

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Yoshida sarcoma cells

The tumour was regularly maintained in Wistar rats by biweekly transplantation of 0.8 ml fluid derived from the intraperitoneal cavity. The ascitic fluid containing Yoshida sarcoma cells was withdrawn from the animals four days after transplantation of this tumour.

Tissue culture cells

The tissue culture cell lines KB (Human oral carcinoma), HeLa (Human cervix carcinoma) were obtained from Virus Research Centre, Poona. These were maintained as monolayers adherent to glass in Eagle's medium modified by Dulbecco. The medium supplemented with 15% human AB serum was replaced twice a week. For use in experiments the bottles were generally seeded with 2.5×10^6 cells and allowed to grow at 37°C for 48 hr. The cells were removed from the glass surface by 0.3% trypsin treatment for 2 min at 37°C.

Protein determination

Protein was assayed by absorbance method described by Kalckar [3].

Preparation of melphalan solution

Melphalan was dissolved in ethanolic HCl–propylene glycol buffer pH 7.2. This was prepared immediately before use.

Assay of cytotoxicity in tissue culture cells

Per cent inhibition of growth of tissue culture

cells by cytotoxin P_6 or melphalan was determined as described [4] except that the cells were grown as monolayers in Leighton tubes. For this purpose 80,000 cells (KB or HeLa) 1 ml of growth medium were seeded in each Leighton tube. Sixteen tubes were used for each group, containing varying amounts of the inhibitor and a control group without the inhibitor. The cells were grown for 48 hr. At the end of this period half the number of tubes in each group were trypsinised and cells counted in a haemocytometer. The medium and the inhibitor in the remaining tubes were replaced and cells were allowed to grow for another 48 hr. The cultures were then trypsinised and counted as before.

The combined effect of cytotoxin P_6 and melphalan on each type of tissue culture cells was studied as follows: four groups each containing 16 Leighton tubes were seeded with 80,000 cells in 0.5 ml growth medium. Groups 1 and 2 were treated with cytotoxin P_6 and incubated for 30 min at 37°C. At the end of incubation period groups 2 and 3 were treated with melphalan. Group 4 was the control to which 10 μl of propylene glycol buffer pH 7.2 used in the preparation of melphalan was added. The final volume of each tube was made to 1 ml with growth medium. The cells were allowed to grow for 48 hr. At the end of this period half the number of tubes of each group was used for counting the cells as before. In the remaining tubes the inhibitors and medium were replaced as described and grown for another 48 hr and the percent inhibition was calculated as above.

Determination of tumour inhibition in rats

Yoshida sarcoma ascitic tumour cells obtained from the intraperitoneal cavity of rats in which the tumour was routinely maintained were inoculated into several groups of male Wistar rats. 1×10^8 cells of tumour inoculum given intraperitoneally to each rat (140–150 g body weight) was found to kill the animal within 8–10 days.

Each experimental group containing four animals was injected i.p. with graded doses of cytotoxin P_6 or melphalan. Each dose was divided into four equal parts and injected at 24 hr intervals. The first injection (i.p.) was given 24 hr after the tumour inoculation. The material was injected in 0.25 ml solution. The control group was injected with 0.25 ml propylene glycol buffer pH 7.2 used to dissolve melphalan.

On the sixth day after inoculation of tumour, the animals were anaesthetised and the ascitic fluid from intraperitoneal cavity was withdrawn and collected in 0.9% saline solution. To collect most of the cells, the intraperitoneal cavity was washed with saline. The total volume was recorded and the percent inhibition of growth of cells in the treated animals was calculated from counts of cells in the control and the experimental groups.

To determine the combined action of cytotoxin P_6 and melphalan on the growth of Yoshida sarcoma cells in rats, the following procedure was adopted. Each dose of cytotoxin P_6 and melphalan was divided into four equal parts and injected at 24 hr intervals as follows: The animals were injected with a dose of cytotoxin P_6 in 0.25 ml distilled water, followed by an injection of a dose of melphalan in 0.25 ml propylene glycol buffer given 4 hr after. The control group was injected with the same volume of propylene glycol buffer. On the 6th day after inoculation of tumour the growth of Yoshida sarcoma cells was determined as described above.

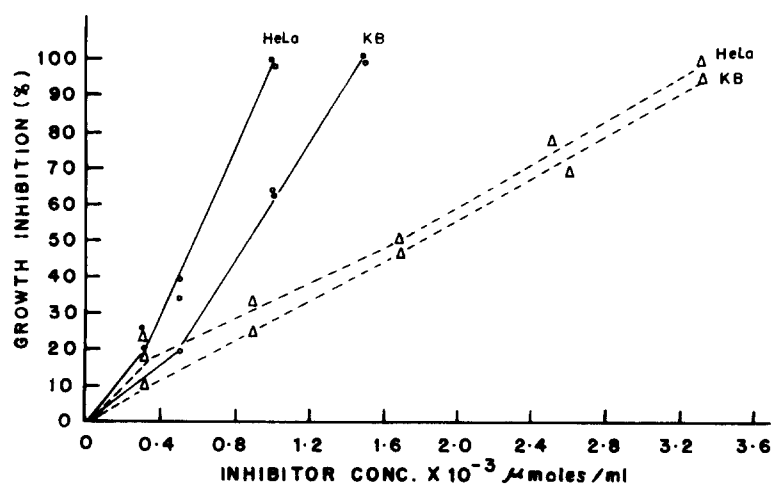

Fig. 1. Growth inhibition produced with varying amounts of cytotoxin P_6 (○—○) and melphalan (△- -△) on HeLa and KB cells.

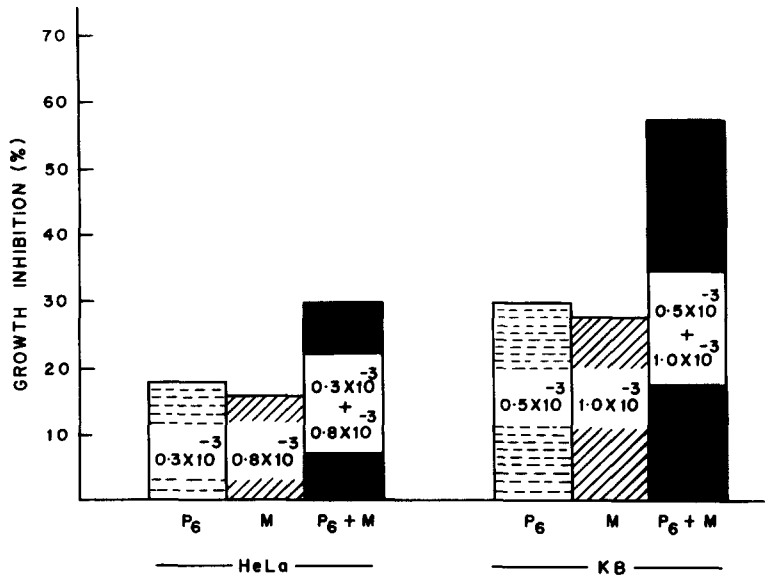

Fig. 2. Effect of simultaneous addition of P_6 and melphalan (M) on HeLa and KB cells. Amounts of inhibitors indicated are μmole/ml *in the system (average of four experiments).*

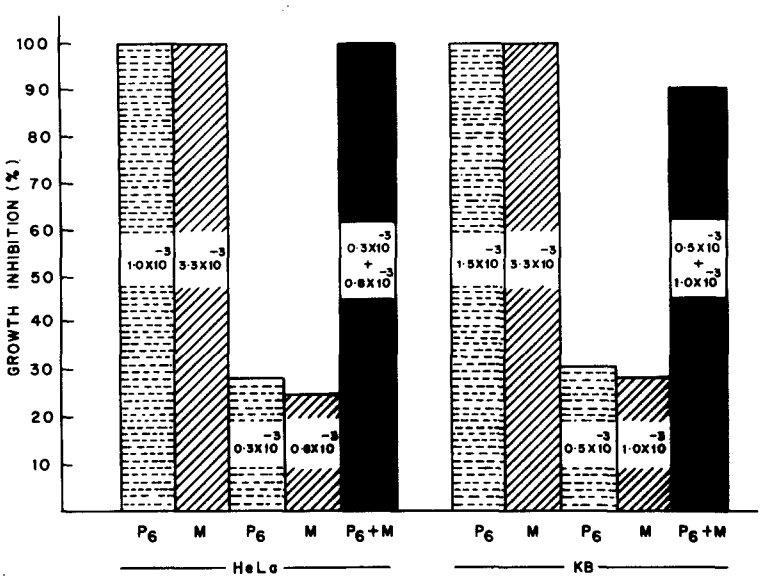

Fig. 3. Potentiating effect of P_6 on melphalan (M) inhibition of growth of HeLa and KB cells. Inhibitor amounts indicated are μmole/ml *in the system (average of four experiments).*

RESULTS

Before examining the composite effects of cytotoxin P_6 and melphalan on HeLa and KB cell lines, the growth of these cells was studied in the presence of varying amounts of each of the inhibitors. Results given in Fig. 1 describe growth inhibition of HeLa and KB cells produced by varying amounts of cytotoxin P_6 and melphalan when added separately. It is seen that 1×10^{-3} μmole/ml of cytotoxin P_6 inhibited the growth of HeLa cells by 100% and 1.5×10^{-3} μmole/ml were required for complete inhibition of KB cells. Melphalan in concentration of 3.3×10^{-3} μmole/ml inhibited by 100% the HeLa cell system and same amount of melphalan produced 95% inhibition of growth of KB cells.

Experiments in which the combined effects of melphalan and cytotoxin P_6 were tested on HeLa and KB cells showed that if the cytotoxin and melphalan were added as a mixture the resultant inactivation was generally additive. It is seen in Fig. 2 that doses of melphalan and cytotoxin P_6 which inhibited the HeLa cells by 16 and 18% respectively, inactivated this cell

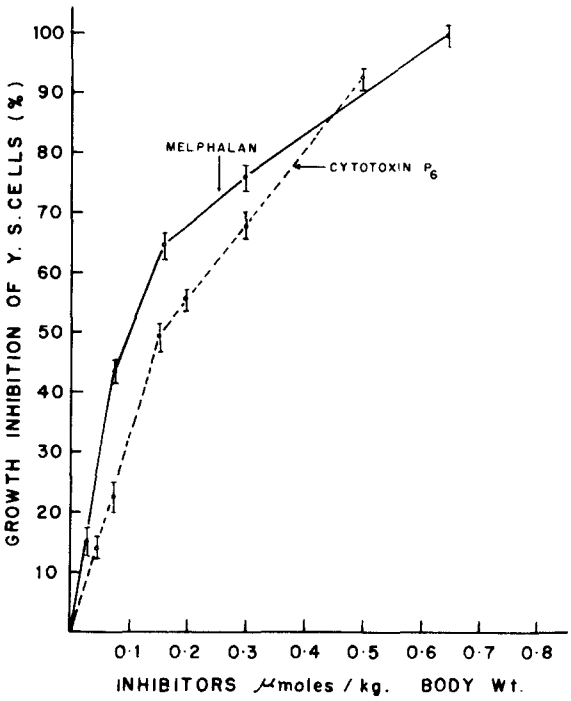

Fig. 4. Percent inhibition of growth of Yoshida sarcoma cells in rats obtained with varying amounts of P_6 and melphalan (M).

system by 30% when added as a mixture. In KB cells a dose of melphalan which by itself inhibited the cells by 28% when mixed with cytotoxin P_6 (a dose which produced 30% inhibition) produced 57% inactivation.

In the next series of experiments attempts were made to facilitate the penetration of melphalan into the cells by their prior exposure to the cytotoxin P_6 which is known to change the membrane permeability [5]. The cells were treated with cytotoxin P_6 and incubated at 37°C for 30 min at the end of which melphalan was added to the experimental tubes. Control tubes containing cells without the inhibitors were treated under identical conditions (see Materials and Methods). Results given in Fig. 3 demonstrate that 0.8×10^{-3} μmole/ml of melphalan when added to HeLa cells primed with 0.3×10^{-3} μmole/ml cytotoxin brought about 100% inactivation of growth. Whereas these quantities of melphalan and cytotoxin when added individually inhibited the cells by 25 and 28% respectively. Similar experiments using KB cells have shown that doses of cytotoxin P_6 (0.5×10^{-3} μmole/ml) which produced 30% inhibition and melphalan (1×10^{-3} μmole/ml) which inhibited these cells by 28% produced 90% inactivation when added sequentially.

Following the observations using tissue culture cells, attempts were made to study the potentiating action of cytotoxin P_6 on melphalan inhibition of cell growth in rats bearing Yoshida sarcoma. Initially the decrease in Yoshida sarcoma cells was determined in the presence of varying quantities of melphalan or cytotoxin P_6. The results obtained with graded doses of melphalan or cytotoxin P_6 ranging from 0.1 to 0.65 μmole/kg body weight are shown in Fig. 4. It was seen that in the control group inoculation of 1×10^8 cells increased to 1×10^9 cells on the 6th day. In the group receiving melphalan, 100% inhibition of cell growth was obtained with 0.65 μmole/kg body weight. Inhibition of 50% was produced with 0.11 μmole/kg body weight. The cytotoxin P_6 produced 94% inhibition with 0.5 μmole/kg body weight and 50% inhibition with 0.15 μmole/kg body weight. To aid the selective penetration of melphalan into the tumour cells, the cytotoxin P_6 was injected 4 hr prior to the injection of melphalan, as described in Materials and Methods. The combined effect was tested with 0.05 μmole/kg body weight of cytotoxin P_6 and 0.04 μmole/kg body weight of melphalan. In these amounts cytotoxin P_6 and melphalan if added separately produced negligible effects on cell growth as compared to the control group (Fig. 4). When the same dose of cytotoxin P_6 was injected i.p. 4 hr prior to melphalan (i.p.), however, the combined inhibition obtained was 65% (Fig. 5). By increasing the dose of melphalan from 0.04 to 0.07 μmole/kg body weight, the inhibition rose to 92%. This concentration of melphalan by itself inhibited the cell growth by 20%.

Animals treated with cytotoxin P_6 and melphalan in doses of 0.05 and 0.07 μmole/kg body weight respectively (Table 1) did not survive for more than 21 days whereas the lifespan of control was 8–10 days. Sequential injection of cytotoxin P_6 and melphalan under same conditions used as above increased the survival time for more than four months. The animals appeared completely normal at the end of this period.

DISCUSSION

Alkylating agents are widely used in human cancer therapy. Like all anticancer agents, they are highly toxic and produce side-effects. A great deal of evidence indicates that the site of action of alkylating agents such as melphalan is in the nucleus brought about by binding to the DNA [6, 7]. As cytotoxin P_6 isolated from cobra venom has been shown to be preferentially destructive to certain tumour cells through binding to plasma membrane constituents [5], the present study was an attempt to determine whether cytotoxin P_6 in minimal doses could enhance the selective penetration of melphalan into the tumour cells and so help to reduce the

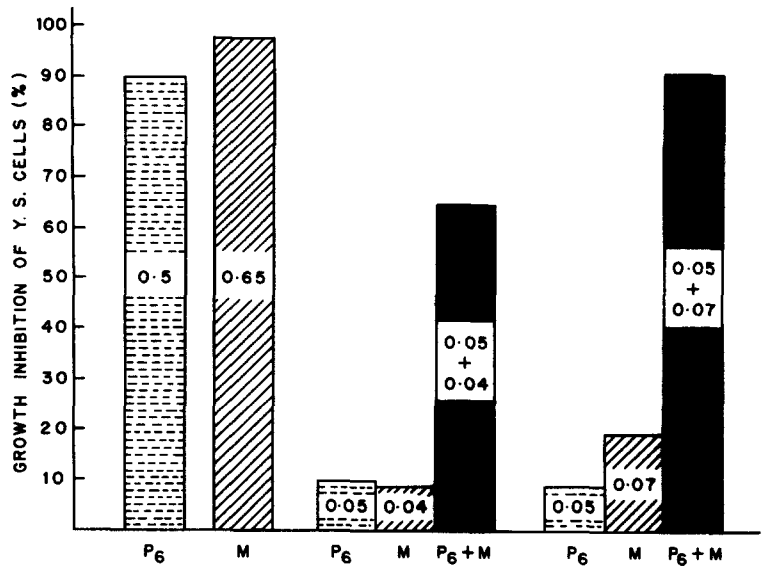

Fig. 5. Potentiating effect of P_6 on the antitumour action of melphalan (M) on the growth of Yoshida sarcoma in rats. Amounts of inhibitors indicated are μmole/kg *body weight. (Average of four experiments).*

Table 1. Lifespan of Yoshida sarcoma rats—control and treated

Sr. No.	group (rats)	Dose of inhibitor (μmole/kg body wt)	Survival (No. of days)
1.	Yoshida sarcoma cells (1×10^8)	—	8–10
2.	Yoshida sarcoma cells (1×10^8) + Cytotoxin P_6	0.05	9–14
3.	Yoshida sarcoma cells (1×10^8) + Melphalan	0.07	8–21
4.	Yoshida sarcoma cells (1×10^8) + Cytotoxin P_6 + Melphalan	0.05 + 0.07	>120

effective dose of this drug. In the *in vitro* experiments with HeLa and KB cells it is seen that when mixtures of melphalan and cytotoxin P_6 were added to the cell system the resultant growth inhibition was additive. This could presumably be due to the slower penetration of the cytotoxic protein into the active sites of cells, as other studies have shown that the cytotoxic action of P_6 on cells is time dependent [8]. However, in experiments in which the HeLa and KB cells were first incubated with cytotoxin P_6 to make them more permeable and then melphalan was added synergistic effects were observed. By the prior priming of the cells with cytotoxin P_6 the effective dose of melphalan required to produce 100% growth inhibition of HeLa and KB cells could be decreased by 75 and 66% respectively. The *in vivo* experiments using Yoshida sarcoma bearing rats demonstrate that when melphalan was injected 4 hr after treatment with cytotoxin P_6, the effective dose of melphalan to produce 100% inhibition of cell growth could be decreased from 0.65 to 0.07 μmole/kg body weight. Experiments in which survival was studied have also indicated that doses of melphalan (0.07 μmole/kg body weight) and cytotoxin P_6 (0.05 μmole/kg body weight) which when tested individually were not able to significantly increase the lifespan of the animal against the tumour prolong the survival to more than four months if injected sequentially as described above. It is interesting to note that on molar basis the cytotoxin P_6 (mol. wt 10,500) appears to be more effective as a growth

inhibitor on HeLa and KB cells than melphalan. Both are approximately of same potency in preventing growth of Yoshida sarcoma in rats. It is also seen that the cytotoxin P_6 displays quantitative differences in its cytotoxicity to HeLa and KB cells. This may be a reflection of differences in the biomembrane structure of these two cell types. Melphalan inhibition was more uniform. The overall data suggests that the cytotoxin P_6, in minute concentration can significantly enhance the antitumour effects of melphalan conceivably by producing preferential changes in membrane permeability of tumour cells.

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In Vivo Studies with Hexamethylmelamine*

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Abstract—*Uptake and distribution studies using* C^{14}*-Hexamethylmelamine in normal and non-tumour bearing mice have shown no selective uptake by the tumour, but a marked concentration of label in the small intestine. Binding of both ring and methyl-*C^{14}*-HMM to liver and to a lesser extent to tumour has been shown to occur. In mice 4 major urinary metabolites were detected. namely* N^2, N^4, N^6*-trimethylmelamine,* N^2, N^4*-dimethylmelamine, monomethylmelamine and melamine. No quantitative difference between HMM and its inactive trimethyl derivative was found in their ability to undergo demethylation* in vivo.

INTRODUCTION

HEXAMETHYLMELAMINE (HMM) has demonstrated consistent activity against a wide range of solid tumours [1]. Of particular interest are the remissions obtained against carcinoma of the lung, which range from 11% for epidermoid carcinomas to 36% for small cell carcinomas [2–5]. Although the efficacy is not particularly striking in any tumour type, clinical interest in HMM continues.

HMM is structurally related to triethylenemelamine (TEM), a known alkylating agent [6], but its metabolism and mechanism of action appear to be completely different [7]. Worzalla *et al.* have shown in both rats and man that the major pathway for metabolism of HMM *in vivo* is *N*-demethylation to lower methylmelamines [8, 9]. HMM is also an effective chemosterilant for house flies [10] and its metabolism in flies has been studied by Chang *et al.* [11], who report that the principal metabolic transformation of HMM again involves *N*-demethylation.

Several suggestions have been made as to how HMM exerts its antitumour activity [12, 13], but the exact mechanism of action remains uncertain. In an earlier paper [14] it was shown that HMM requires metabolic activation in order to exert its antitumour effects *in vitro* and that *N*-methylols may well represent the active form of the drug. Two such derivatives have shown activity against PC6 plasma cell tumour *in vivo* and very marked activity against a human lung tumour (P246 epidermoid carcinoma of the bronchus) growing in immune deprived mice [15], in common with the parent compound.

In the present paper a study of the uptake and tissue distributions of C^{14}-HMM in normal mice and in mice bearing tumours sensitive to HMM is reported. The binding of both ring and methyl labelled HMM *in vivo* has also been looked at in an attempt to ascertain if the activity of these methylols is inherent, or whether they act as formaldehyde releasers, which is itself the tumour-active agent.

A method for measuring blood levels of formaldehyde produced as a result of the demethylation of HMM and its analogues is also described. Finally the urine metabolites from mice treated with a tumour-curative dose of HMM have been examined.

MATERIAL AND METHODS

Chemicals

2,4,6-C^{14}-Ring HMM, specific activity 32 mCi/mmole, was prepared at Services des

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Molecules Marquees C.E.N., Saclay, France, from ^{14}C-urea by a 2-step synthesis. ^{14}C-urea was cyclized in *o*-dichlorobenzene to (2,4,6-^{14}C) cyanuric acid, purified by chromatography on an ion exchange resin column Sephadex SPC-25. Direct amination of acid in presence of HMPT and dimethylamine gave 2,4,6-C^{14}-Ring HMM, purified by silicagel adsorption column chromatography; radioactive overall yield: 18% based on ^{14}C-urea [16].

2,4,6-N($^{14}CH_3$)$_2$-HMM, specific activity 8.4 mCi/mmole, was generously provided by the Stanford Research Institute, Menlo Park, California.

Unlabelled HMM was provided by the National Cancer Institute, Bethesda.

Animals and tumours

Female CBA/LAC mice were used for normal studies. The P246 human bronchial carcinoma was implanted as tumour pieces s.c. into the flank of immune-deprived male CBA/LAC mice according to the method of Mitchley *et al.* [17]. The tumours were used 8–9 weeks after implant when they had grown to a weight of approx. 3 g.

The ADJ/PC6 plasma cell tumour was obtained by s.c. transplantation of tumour fragments in the flank of female BALB/c mice and used 24 days after transplantation, i.e. when antitumour testing is normally started.

Measurement of blood levels of formaldehyde

Groups of 5 normal CBA mice were treated with a tumour-curative dose (90 mg/kg) of the drug under study, injected intraperitoneally as a suspension in arachis oil. At various time intervals from 0 to 4 hr after injection the animals were killed using solid carbon dioxide and the blood collected after snipping the heart. Blood samples taken were added to a solution containing semicarbazide (1.11 mg/ml) kept on ice and deproteinised immediately by the addition of 1 vol sodium tungstate followed by 1 vol 0.67 ml H_2SO_4. The deproteinised samples were kept on ice for approx. 15 min and the protein precipitate then removed by centrifugation. The resulting supernatant was neutralized by adding 0.0167 vol of 0.67N NaOH and formaldehyde estimated according to the method of Nash [18].

Uptake and distribution of C^{14}-HMM

C^{14}-Labelled HMM was administered to mice (2.4 mg/kg i.p. in ETOH/oil) and at various time intervals after injection the major organs and tumour were removed. The larger organs, namely liver, tumour, small intestine and large intestine, were homogenized in distilled water (1 g wet weight tissue to 4 ml water) and a 1 ml aliquot of homogenate digested by heating with 1 ml 25% tetraethylammonium hydroxide (TEH) at 50°C overnight. One tenth of a millilitre of the digest was then added to 10 ml scintillation fluid and counted in a scintillation counter (Packard Tricarb Model 3380).

The smaller organs were solubilized using Soluene 350 (100 mg/ml) 0.1–0.25 ml aliquots of each digest were then taken for scintillation counting as above.

Binding of C^{14}-HMM

The binding of ring and methyl-labelled HMM to the TCA-insoluble fraction of liver, tumour and small intestine was measured using aqueous tissue homogenates prepared from organs of mice treated with C^{14}-HMM as described above.

A 1.0 ml aliquot of homogenate was taken and an equal volume of 10% trichloroacetic acid (TCA) was added to precipitate protein and nucleic acids. The centrifuged material was redissolved in 2 ml TEH by heating overnight at 50°C to give a measure of the amount of bound label. The supernatant was used to measure free label. Samples were assayed for radioactivity by taking an aliquot (100 µl) in 10 ml scintillation fluid and counting as before.

Urine metabolites

A group of 10 non-tumour bearing CBA/LAC mice were treated with 90 mg/kg HMM, consisting of 1 mg ring-C^{14}-HMM: 9 mg unlabelled drug and urine samples collected every 24 hr for 3 days. The urine samples were purified by column chromatography according to the method of Worzalla *et al.* [8], and subjected to t.l.c. analysis on MN Cel 300–10 cellulose plates using dimethylamine/methanol/H_2O (4:9:2) as developing solvent.

RESULTS

Formaldehyde blood levels

It has been possible to detect a Nash positive metabolite in the blood of mice treated with HMM and its analogues. It is uncertain as to whether formaldehyde is actually being measured directly in this system or indirectly from methylol extracted with the blood, which would then break down spontaneously under the assay conditions employed since this involves heating to 60°C for 30 min. In either case, this technique

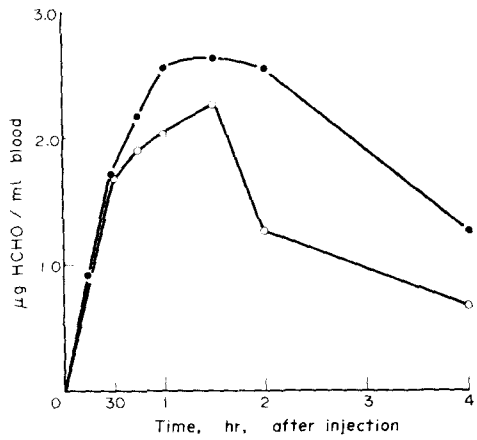

Fig. 1. Formaldehyde levels in blood following treatment with hexamethylmelamine (90 mg/kg) *and trimethylmelamine* (90 mg/kg). [*Figs. 1 and 2, each point represents mean value for 5 mice*].

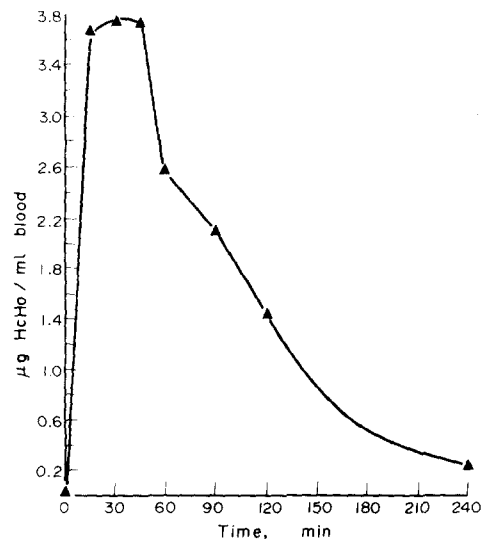

Fig. 2. Formaldehyde levels in blood following treatment with pentamethylmonomethylolmelamine (90 mg/kg).

may be usefully employed as an index of metabolism *in vivo*.

From Fig. 1 it can be seen that following administration of HMM the level of formaldehyde in the blood rises sharply reaching a maximum of 2.6 µg HMM/ml blood at 90 min and then falls again less steeply. A second experiment using trimethylmelamine, which has been shown to be inactive as an antitumour agent, shows a similar curve again reaching a peak after 90 min, except that the level of formaldehyde is slightly lower, i.e. 2.3 µg/ml blood and there is a sharp initial decrease in blood formaldehyde.

Figure 2 shows a similar type of experiment using the monomethylol derivative of HMM (CB10-369), which is the postulated intermediate in the primary demethylation of HMM. In this case, the blood level of formaldehyde is seen to rise even more sharply than for either HMM or TMM, reaching a maximum after about 30 min or possibly earlier when the level of formaldehyde is equal to 3.7 µg/ml blood, i.e. somewhat higher than for the two methylmelamines. The blood level of formaldehyde also falls away very sharply for PMM-methylol so that after 4 hr very little formaldehyde is detectable.

Uptake and distribution of C^{14}*-HMM*

Using ring-C^{14}-HMM in normal mice it is clear that the excretory organs, liver and kidney, showed high concentrations of label (Fig. 3), while only relatively low levels of label were found in organs such as muscle and brain. However, one very interesting feature is the marked concentration of label in the small intestine of these mice which is 2–3 times higher than in either liver or the corresponding large intestine.

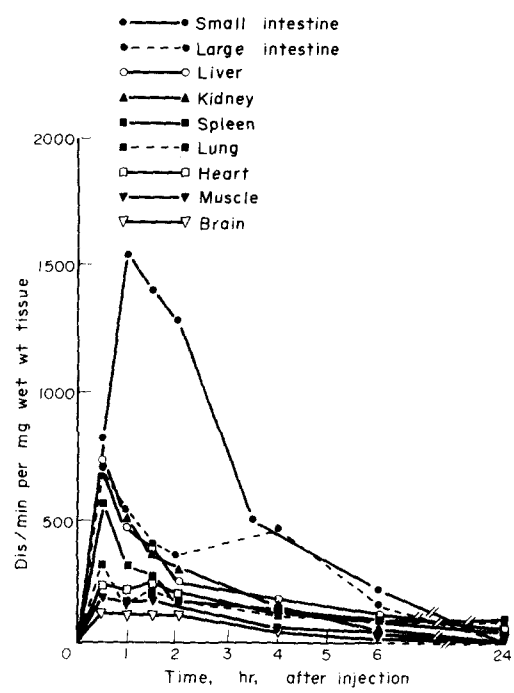

Fig. 3. Distribution of ring-C^{14}-Hexamethylmelamine in normal mice.

Using ring-C^{14}-HMM in PC6 tumour bearing mice (Fig. 4) it can be seen that there is no selective uptake of the drug by this particular tumour (tumour: liver ratio 0.48) despite its sensitivity, but again there is an especially high localization of radioactivity in the small intestine which is three times greater than the large intestine concentration.

The same observations apply to mice bearing the P246 human tumour xenograft (Fig. 5) although here uptake of drug by the tumour is

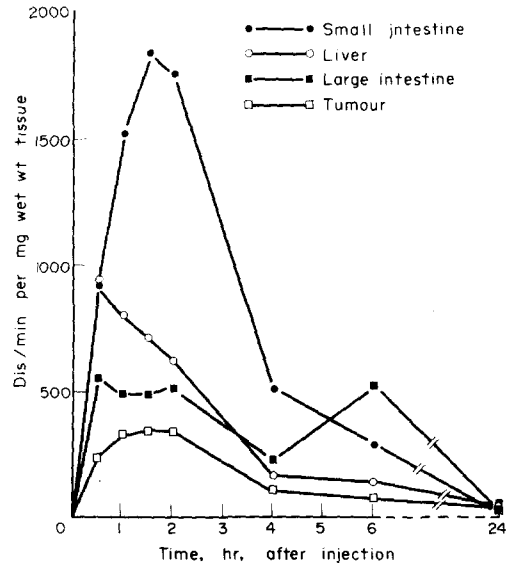

Fig. 4. Distribution of ring-C^{14}-HMM in PC6 tumour-bearing mice.

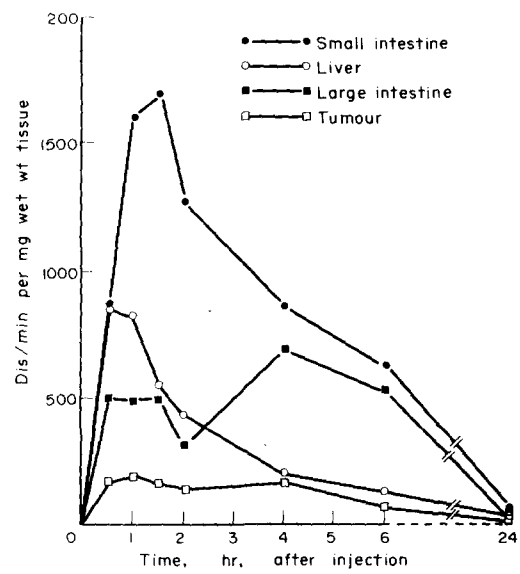

Fig. 5. Distribution of ring-C^{14}-HMM in P246 human tumour-bearing mice.

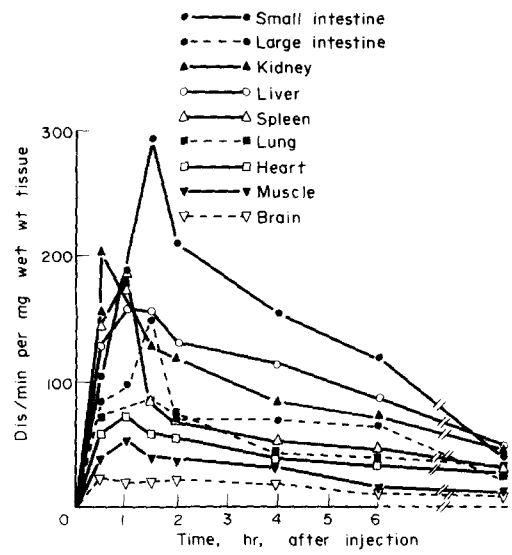

Fig. 6. Distribution of methyl-C^{14}-HMM in normal mice

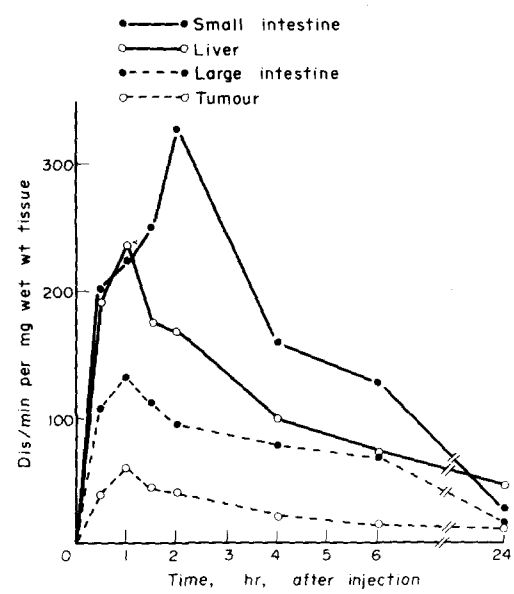

Fig. 7. Distribution of methyl-C^{14}-HMM in PC6 tun bearing mice.

even lower (tumour: liver ratio = 0.23), although this low level of drug appears to be maintained over a longer period of time compared with other organs, as is also seen to a lesser extent with the PC6 tumour. Generally uptake of ring-C^{14}-HMM is rapid in all organs, reaching a maximum at about 30 min and declining almost equally rapidly.

Using methyl-C^{14}-HMM (Fig. 6) uptake of label is slightly slower in most organs with maximum levels reached after about 60 min and there is a corresponding slower fall in radioactivity. The overall tissue distribution for the methyl labelled material is much the same as for ring-C^{14}-HMM with again this large concentration of label in the small intestine. This is found consistently in both PC6 (Fig. 7) and P human tumour bearing mice (Fig. 8), with every case a three fold difference between sı and large intestine concentrations. The ratio for methyl C^{14}-HMM in both tun types is again of the order of 0.24–0.26, cle demonstrating non-selective uptake.

Binding studies

Figure 9 shows that binding of both ring-methyl-C^{14} HMM to liver occurs in no mice. Initially there is little difference ir degree of binding between the two types of l but after 24 hr binding of the ring-C^{14} pound is significantly higher than the m

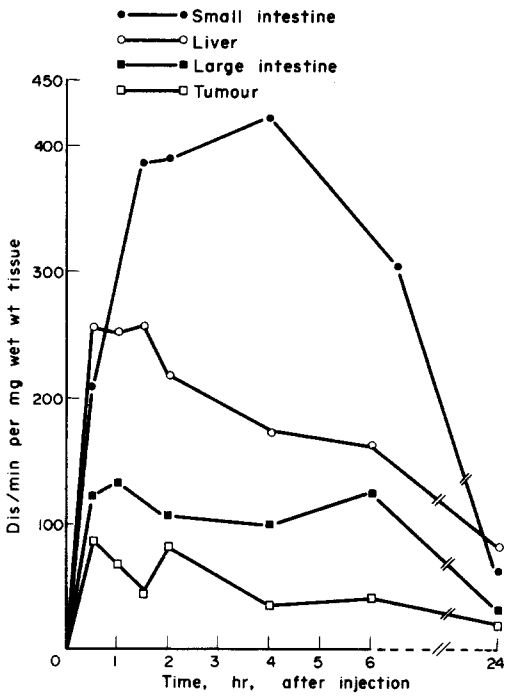

Fig. 8. Distribution of methyl-C^{14}-HMM in P246 human tumour-bearing mice.

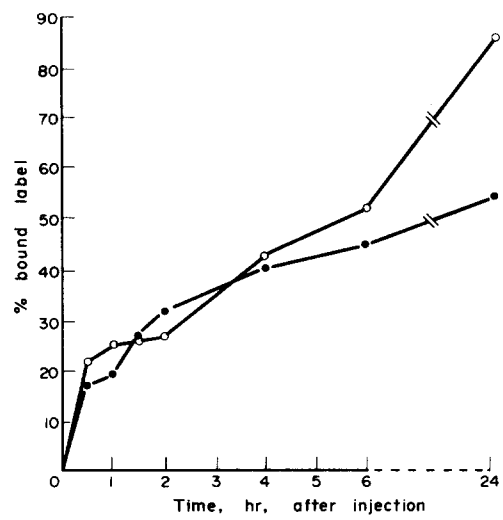

Fig. 9. Binding of ring- and methyl-C^{14}-HMM to TCA-insoluble fraction of liver in normal mice.

labelled drug, i.e. 88 as opposed to 50% bound label. Although the percentage of drug in the TCA-insoluble fraction increased with time the actual amount of bound material remained more or less constant.

In PC6 tumour bearing mice (Fig. 10) binding of both types of label was found to be higher in liver than in tumour, but binding of methyl-C^{14}-HMM is greater than for ring-C^{14}-HMM in both organs in these animals.

In mice bearing the human bronchial tumour the same situation occurs, i.e. that tumour binds

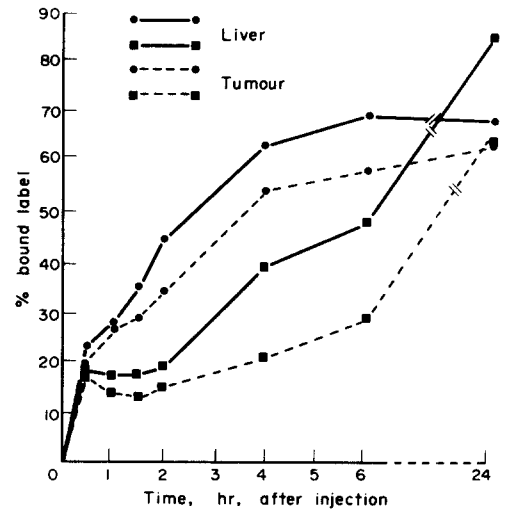

Fig. 10. Binding of ring- and methyl-C^{14}-HMM to TCA-insoluble fractions of liver and tumour in PC6 tumour-bearing mice.

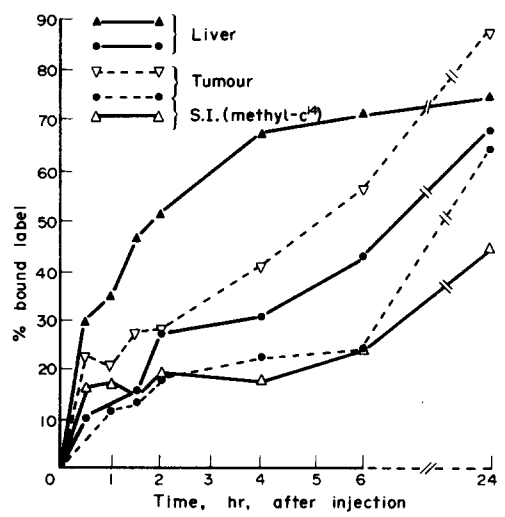

Fig. 11. Binding of ring- and methyl-C^{14}-HMM to TCA-insoluble fraction of liver, tumour and small intestine in P246 human tumour-bearing mice. [Figs. 3–11, each point represents mean value for at least 2 mice].

both types of labelled drug to a lesser extent than liver and the situation for normal mice is again reversed in that methyl-C^{14} binding is greater than ring-C^{14} binding for both liver and tumour tissue (Fig. 11). Figure 11 also shows that despite the high concentration of methyl-C^{14} label in

Table 1.

Time (hr)	% Total dose of radioactivity recovered in urine (2.9×10^8 dis/min)
24	81.9
48	6.94
72	1.07
Total over 3 days = 89.9	

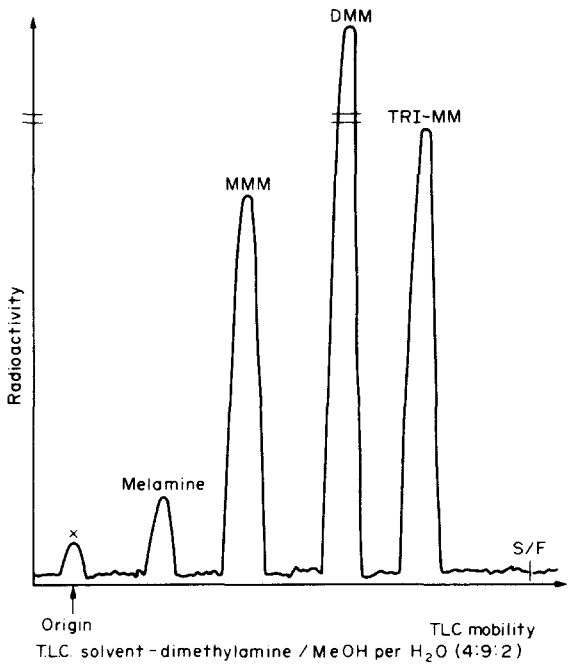

Fig. 12. Thin-layer radiochromatogram of ring-C^{14}-HMM metabolites in 24 hr mouse urine. MMM, monomethylmelamine; DMM, dimethylmelamine; Tri-MM, trimethylmelamine.

Table 2.

Compound	R_F	% Total radioactivity
Trimethylmelamine	0.80	20.0%
Dimethylmelamine	0.66	74.6%
Monomethylmelamine	0.49	4.5%
Melamine	0.36	0.61%
X	0	0.18%

the small intestine, the amount of bound material is relatively small compared with liver and tumour.

Table 1 shows that when mice were treated with ring-C^{14}-HMM at a tumour curative dose almost 90% of the injected radioactivity could be found in the urine and that the vast majority of this radioactivity was found in the first 24 hr sample.

Thin-layer chromatographic analysis of the purified urine sample revealed the presence of 4 major metabolites of HMM (Fig. 12). These have been identified as N^2, N^4, N^6 trimethylmelamine, N^2, N^4-dimethylmelamine, monomethylmelamine and melamine (Table 2). Thus dimethylmelamine is the major methylmelamine metabolite. A small unidentified peak of radioactivity was also found at the origin.

When the acidified urines (0.1N) were added to the Dowex (Cl^-) column and eluted with 0.1N HCl, 98% of the radioactivity was recovered. Total radioactivity recovered following 2.4N and 6N HCl wash of Dowex 50W (H^+) column in the second stage of purification was only 89%.

DISCUSSION

It has been suggested that HMM may exert its antitumour activity through formaldehyde release [12]. Assuming this theory to be correct from the formaldehyde blood level studies reported here it is difficult to explain the difference in antitumour activity between HMM and trimethylmelamine. Both compounds have been shown to produce similar levels of formaldehyde over more or less the same time course and yet HMM has shown good activity against both the PC6 mouse tumour and human bronchial tumour, whereas the trimethyl derivative is quite inactive in both test systems [14, 15]. It may be argued that, if formaldehyde is the active metabolite, the rate of release of this agent is the determining factor, since the level of formaldehyde produced by HMM is seen to fall more slowly than for trimethylmelamine, or less likely that the final level of formaldehyde obtained is critical in determining whether or not a compound has antitumour activity.

However, both these explanations are unlikely and a better explanation of these findings can be made assuming that *N*-methylols represent the active metabolite. In this case a structural difference between the methylols formed by HMM and tri-MM may explain their differences in activity, or perhaps a difference in stability between the respective methylol intermediates.

When the monomethylol of HMM is looked at in this system the sharp rise and fall in blood level formaldehyde produced by this compound is consistent with its being an intermediate in the metabolism of HMM in which the rate determining step is in the initial hydroxylation, followed by a more rapid breakdown to formaldehyde. These findings provide a reasonable explanation for the *in vivo* antitumour tests using the PC6 tumour [14] in which the two methylols were found to have a lower therapeutic index than the parent compound. When HMM is the starting compound, levels of the active metabolite may be maintained over a period of time, whereas methylols are unstable and will break down fairly rapidly *in vivo*.

Support for the theory that an *N*-methylol rather than formaldehyde represents the active metabolite comes from binding experiments with ring- and methyl-C^{14}-HMM. It is of interest that in tumour tissue ring-labelled

HMM is found to bind to a high degree to cellular macromolecules, indicating that the complete molecule is binding and not only metabolized methyl groups, as would be the case if only methyl-C^{14}-HMM was found to bind.

Binding studies with C^{14}-HMM have also shown an apparent difference between normal and tumour-bearing mice in their ability to bind the ring- and methyl-labelled drug. In both types of tumour bearing mice the fact that binding of methyl-C^{14}-HMM is greater in liver and tumour than for the ring-C^{14} compound suggests either increased metabolism of the drug by tumour-bearing mice, a different type of metabolism, or alternatively a different site of binding.

The relatively poor uptake of labelled HMM by tumour compared with other organs is not surprising in view of the fact that many effective antitumour agents, with the exception of some sulphonamidetriazenes, are not selectively concentrated by tumour cells [19]. That high drug uptake is not necessarily important for antitumour effect is shown by the relatively poorer uptake by the xenografted tumour compared with the mouse plasma cell tumour, the latter being much less sensitive.

The very marked concentration of radioactivity found in the small intestine and correspondingly low level in the large intestine has led to the suggestion that HMM is excreted in the bile, with the possibility of entero-hepatic circulation. Other experiments have indicated that this does appear to be the case, and further that conjugation of the drug to form a glucuronide [20] may be occurring, e.g. an *o*-glucuronide of the methylol. Of interest in this respect is the finding that those tumours sensitive to HMM possess high levels of the lysosomal enzyme β-glucuronidase. An explanation of the selectivity of this drug may therefore be found in the selective breakdown of a glucuronide derivative of HMM by such tumour cells.

Using isolated liver microsomes it was found that demethylation of trimethylmelamine *in vitro* does not occur to any significant extent [14]. From the *in vivo* experiments in which blood formaldehyde is measured, however, HMM and tri MM appear to be equally well demethylated. Furthermore, if HMM is demethylated *in vitro* using a 10,000 *g* supernatant metabolizing system an increase in metabolism of the order of 30% is observed, and lower methylmelamine metabolites can be isolated, suggesting perhaps that soluble factors are also involved in the oxidative demethylation of this compound.

These findings are supported by an examination of the urine metabolites of ring-C^{14}-HMM, since only lower methylmelamines were found, predominantly dimethylmelamine, although some melamine was also detected. There was no evidence of higher methylmelamines being present so that in mice HMM must be rapidly and completely demethylated. The unidentified minor urine metabolite X may represent a glucuronide conjugate of an HMM metabolite.

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Letter to the Editor

Antitumour Effect of Cyclofenil (F6066) on DMBA-Induced Rat Mammary Tumors*

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AMONG synthetic non steroid weak estrogens, cyclofenil [1] (F6066, Fig. 1) is a substance having also a weak antiestrogenic activity (about 1/400 of clomiphene) [2]. Although its mode of action is unknown, some of the physiological effects of the drug might be mediated through estrogen receptors since it was reported to bind with a rather high binding affinity to the cytoplasmic uterine receptors [3, 4] as all other antiestrogenic–estrogenic substances. A tridimentional conformation analysis suggested that the affinity of the drug might be due to geometrical similarities with estradiol or diethylstilbestrol [4]. Since strong antiestrogens (U11, 100A; CI 628; ICI 47,464 . . .) were shown to be active in the treatment of mammary carcinomas, it was of interest to evaluate whether a weak antiestrogen such as cyclofenil with a somewhat higher binding affinity for the receptors than strong antiestrogens [3] might also be effective.

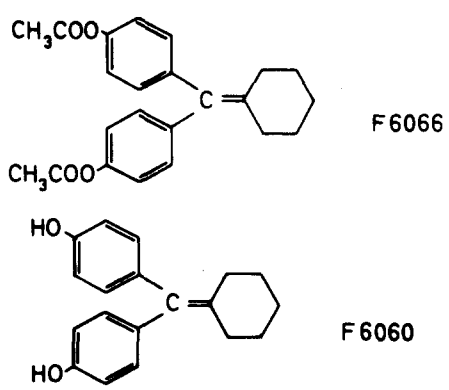

Fig. 1. Formula of cyclofenil (F6066) and its deacetylated form (F6060).

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§This service is a member of the European Organisation for Research on Treatment of Cancer (E.O.R.T.C.).

The dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumor system is a well known model to evaluate the potential antitumor efficiency of antiestrogenic–estrogenic substances. Evidence was reported indicating that tumors responding to these substances contain significant amounts of estrogen receptors. The biological characteristics of cyclofenil led us to evaluate its antitumor activity on this model. The binding affinities of the drug and of its deacetylated form (F6060; Fig. 1) for the tumor estrogen receptors were also analysed.

Mammary tumors were induced in female rats, aged 50 days, by a single i.v. injection of a lipid emulsion containing 5 mg DMBA. Tumor cytosol was prepared in 10 mM Tris–HCl buffer pH 7.5 containing 3 mM EDTA and 6 mM β-mercaptoethanol as previously described [5]. The ability of F6066 and F6060 to bind to the tumor cytosol estrogen receptors was tested by inhibition of the binding of ^{3}H-estradiol. This ability was compared with that of unlabeled estradiol. Cytosol fractions were incubated with a saturating concentration of ^{3}H-estradiol (6×10^{-9} M) (115 Ci/mmole; The Radiochemical Centre, Amersham, U.K.) in the absence or presence of increasing amounts of a drug or unlabeled estradiol (Sigma Co, St Louis, Mo). An inhibition curve was observed in the presence of each competitor (Fig. 2). Fifty per cent inhibition was achieved at the following concentrations: unlabeled estradiol, 7×10^{-9} M; F6060, 10^{-7} M and F6066, 5×10^{-7} M. These values suggest binding affinities roughly 14 and 70 times lower than estradiol for F6060 and F6066 respectively. It is of interest that the affinity of the deacetylated form of the drug (F6060) was slightly higher, which suggests that (a) phenolic group(s) of the molecule participate(s) in its binding to the receptors as in the case of estradiol. Exchange assays [6] showed that bound drugs were displaced from the receptors by ^{3}H-estradiol indicating a reversible binding.

Table 1. Effect of F6066 on growth of established tumors

Group	Mean total tumor surface per rat (mm^2) during treatment					
	Start	2 weeks		9 weeks	6 weeks†	
Control	276	447	(+171)*	654 (+378)	993	(+717)
A	236	388	(+152)	506 (+270)	671	(+435)
B	250	407	(+157)	495 (+245)	529	(+279)
C	228	292	(+64)	268 (+40)	265	(+37)

*Increase of tumor surface.
†Statistical significance: A vs control: ns, B vs control: ns, C vs control: $P<0.02$ (Wilcoxon test on the paired differences).

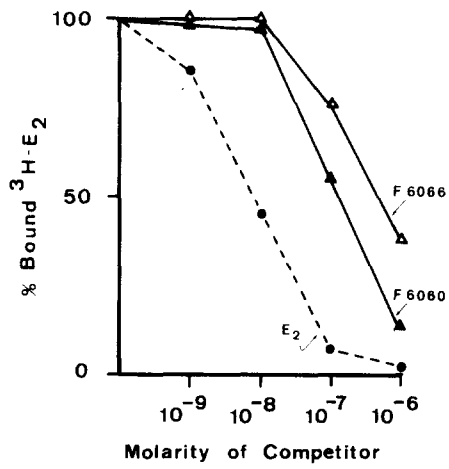

Fig. 2. Inhibition of the binding of ^{3}H-estradiol (^{3}H-E_2) to DMBA-induced rat mammary tumor cytosol by unlabeled competitors: estradiol (E_2), F6066 or F6060. Aliquots of 100 µl of cytosol were incubated overnight at 4°C with an equal volume of buffer containing ^{3}H-E_2 at the final concentration of 6×10^{-9} M in presence or absence of increasing amounts of an unlabeled competitor. Bound ^{3}H-E_2 was then measured after extraction of unspecifically bound components by the dextran-coated charcoal method [7].

The effect of F6066 on the growth of established tumors was analyzed. Tumor-bearing rats were matched into "blocks" according to the number and sizes of the tumors, the latter being expressed as "surface", that is the product of two perpendicular diameters as measured by means of a caliper. The animals of each block were randomized into 4 groups of 12 rats each and were subjected to daily subcutaneous injection for 6 days per week during 6 weeks. Groups A, B and C received respectively 0.1, 1 and 10 mg/kg F6066 suspended in 0.1 ml sesame oil; the control group received sesame oil only. The rats were weighed weekly and their tumor surface was measured every two weeks. Treatment effects were estimated by the differences of total tumor surface (sum of individual tumor surface) between matched rats. Results are reported in Table 1. After 2, 4 and 6 weeks of administration at each dose, F6066 induced a reduction of tumor growth as compared to the controls. At the end of experiment the reduction observed at 10 mg/kg was significant ($P<0.02$; Wilcoxon test on the paired differences) whereas the lower doses were not. A small decrease in gain of body weight was recorded (per cent weight gain in, controls: +6, group A: +4, group B: +2, group C: −3). This difference was non-significant ($P>0.1$, Mann–Whitney U test) thus suggesting that the reduction of tumor growth was not due to a systemic toxic effect of the drug and resultant weight loss.

Vaginal smears, taken daily, showed irregular estrus cycles in controls and treated rats. At the end of the experiment the animals were sacrificed. A reduction of the weight of the ovaries was recorded (percentage of weight loss as compared to the controls; group A: −15, group B: −20, group C: −40). There was a similar reduction in uterine weight (percentage loss as compared to the controls, group A: −9, group B: −14, group C: −21). Histological examination of the ovaries from the rats receiving 10 mg/kg revealed the presence of numerous atretic follicles; no corpora lutea were found. In contrast, controls showed a normal ovarian histology. All these data are consistent with the reported weak antiestrogenic activity of F6066 [1, 2].

The present data demonstrate that cyclofenil is an effective antagonist of the growth of the DMBA-induced mammary tumors of the rat. In contrast to all presently tested antiestrogens, the drug produces its antitumor effect without detectable modification of the estrus cycle; all other antiestrogens were reported to induce a permanent diestrus. These results indicate that a strong antiestrogenic activity is not required to interfere with tumor growth and suggest, thereby that weak antiestrogens might be useful for the treatment of human breast cancer. It may be speculated however that a strong enough binding affinity for estrogen receptors might be a prerequisite for antitumor activity.

Acknowledgements—We wish to thank the Ferrosan Co. (Malmö, Sweden) for the gift of F6066 and F6060 as well as for a financial support. We also thank Dr. P. E. Schurr (the Upjohn Co, Kalamazoo, Mich.) for the preparation of DMBA Emulsion. Finally, the technical assistance of Mrs. G. Wijnands was highly appreciated.

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Announcement

N.C.I.–E.O.R.T.C. SYMPOSIUM ON NEW DRUGS IN CANCER THERAPY

To be held on 7–8 September, 1978 at the Institut Jules Bordet, Brussels.

Chairmen: F. MUGGIA and H. J. TAGNON

September 7: E.O.R.T.C. and N.C.I. Investigational Drugs
September 8: Adriamycin and Anthracycline Derivatives

A small number of free communcations will be integrated in the program. Abstracts must be sent to Dr. M. Rozencweig, Division of Cancer Treatment, National Cancer Institute, Bldg 37, Rm 6A17, Bethesda, Maryland 20014. U.S.A. *before* 15 *July*, 1978.

Among the participants:

P. ALBERTO (Geneva), N. BACHUR (Baltimore), L. BAKER (Detroit), R. BENJAMIN (Houston), G. BONADONNA (Milan), S. K. CARTER (Palo Alto), M. CASAZZA (Milan), H. CORTES-FUNES (Madrid), J. DANIELS (Stanford), H. DAVIS (Madison), S. GARATTINI (Milan), M. GHIONE (Milan), A GOLDIN (Bethesda), H. HANSEN (Copenhagen), D HENRY (Research Triangle Park), Y. KENIS (Brussels), G. MATHÉ (Paris), F. MUGGIA (Bethesda), C. MYERS (Bethesda), C. PRAGA (Milan), M. ROZENCWEIG (Bethesda), M. STAQUET (Brussels), H. TAGNON (Brussels), A. TROUET (Brussels), D. VON HOFF (Bethesda).

Number of participants will be limited to 200.

For details and registration, write to Dr. M. Staquet, E.O.R.T.C. Data Center, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Bruxelles, Belgium. (Phone: 538.57.90—Telex: 22773).

The symposium will be held 2, rue Evers (1st floor), 1000 Bruxelles. Registration fee: 3000 Belgian francs.

THE NETHERLANDS CANCER INSTITUTE (ANTONI VAN LEEUWENHOEK-HUIS) INTERNATIONAL CONFERENCE ON "ENVIRONMENTAL CARCINOGENESIS: OCCURRENCE. RISK EVALUATION AND MECHANISMS OF ACTION OF CHEMICAL CARCINOGENS"

On the occasion of the opening of the new Research Laboratory of the Netherlands Cancer Institute an international congress will be held 7–11 May, 1979, at the RAI-Congress Centre in Amsterdam.

Some 20 invited speakers will discuss the epidemiology of cancer, occurrence of carcinogens and mechanisms of their action, transplacental carcinogenesis; Host factors as carcinogen metabolism and DNA-repair; Modifying factors: dietary components, enzyme inducers, promoters, anticarcinogens; Evaluation of animal experiments and risk assessment of low doses of carcinogen in large populations; Evaluation of short-term assays for identifying chemical carcinogens.

At the end of each daily session a period of one hour is reserved for open discussion.

For further information, write: Dr. E. Kriek, secretary Organizing Committee, The Netherlands Cancer Institute (Antoni Van Leeuwenhoek-Huis) Plesmanlaan 121, Amsterdam-Slotervaart, The Netherlands.

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Recent Journal Contents (1978)

International Journal of Cancer — *May, 1978*

Human Cancer

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S. R. Waldman and R. H. Yonemoto: The use of cryopreserved lymphocytes in the leukocyte adherence inhibition assay. I. Evaluation of specificity of responses in cancer patients.

G. Klein, E. Yefenof, K. Falk and A. Westman: Relationship between Epstein–Barr virus (EBV)-production and the loss of the EBV receptor/complement receptor complex in a series of sublines derived from the same original Burkitt's lymphoma.

T. Godal, A. Henriksen, J.-G. Iversen, T. Ø. Landaas and T. Lindmo: Altered membrane-associated functions in chronic lymphocytic leukemia cells.

N. Imamura, T. Takahashi, K. O. Lloyd, J. L. Lewis, Jr. and L. J. Old: Analysis of human ovarian tumor antigens using heterologous antisera: detection of new antigenic systems.

E. Murray, S. Ruygrok, G. W. Milton and P. Hersey: Analysis of serum blocking factors against leukocyte dependent antibody in melanoma patients.

B. M. Vose, F. Vánky, M. Fopp and E. Klein: *In vitro* generation of cytotoxicity against autologous human tumour biopsy cells.

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E. Gorelik, S. Segal and M. Feldman: Growth of a local tumor exerts a specific inhibitory effect on progression of lung metastases.

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M. A. Basombrio and R. Laguens: Active immunization against murine radiation-induced leukemia using a sarcoma virus pseudotype.

C. J. Wikstrand, J. O. Proctor, S. C. Hartsell and G. Haughton: Murine Rous sarcoma specific immunity detected by leukocyte adherence inhibition: inhibitory effect of normal serum.

G. Vlahakis, H. C. Chopra and M. R. Sawdon: Induction of mammary tumors in C57BL/Haag female mice by a low oncogenic mammary tumor virus.

L. Falk, D. Johnson and F. Deinhardt: Transformation of marmoset lymphocytes *in vitro* with Herpesvirus ateles.

Y. Yoshikawa, K. Yamanouchi, M. Hishiyama and K. Kobune: Lysis of RSV-transformed Japanese quail cells by a factor from normal quail serum.

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Isozyme Pattern of Pyruvate Kinase During Hepatocarcino-Genesis Induced by 2-Acetylaminofluorene in Rat Liver*

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Abstract—*The biosynthesis of pyruvate kinase isozymes has been studied in rat liver during the administration of carcinogen 2-acetylaminofluorene. Transition from a predominance of isozyme L to K was observed during hepatocarcinogenesis. Changes in isozymic pattern and specific activity of pyruvate kinase were observed before histological alterations typical for cancer formation in rat liver could be detected. The dedifferentiation during hepatocarcinogenesis results in switching on the gene for isozyme K and switching off the gene for isozyme L. Within a single liver cell, however, both genes are not functioning simultaneously. Metabolic consequences of the observed changes are discussed.*

INTRODUCTION

THREE distinct isozymes of pyruvate kinase (PK) named L, K and M have been detected in mammalian tissues. Each of these isozymes consists of four identical or almost identical polypeptide chains [1, 2]. It has been demonstrated in several laboratories that isozymes of pyruvate kinase differ in electrophoretic mobility [3–7], isoelectric point [1, 8, 9], kinetics [10–14] and immunological properties [15, 16]. The presence of both divalent and monovalent cations is necessary for pyruvate kinase activity [17, 18].

L type isozyme of PK has been found in hepatocytes, intestine, kidney [19–21] and it probably also occurs in erythrocytes [6, 22]. This isozyme plays an important role in regulation of glycolysis and gluconeogenesis [5, 23, 24]. L type PK displays cooperative kinetics with respect to phosphoenolpyruvate [10, 14, 25] and its activity is inhibited by various metabolites: ATP, L-alanine, L-proline, L-valine and L-phenylalanine [13, 15, 26, 27]. Fructose-1, 6-bisphosphate changes its kinetics from sigmoidal to hyperbolic [22, 28, 29]. It has been suggested that isozyme L is present in two interconvertible forms which differ in their kinetic characteristics [22, 30]. Recently, L type of PK has been separated into three activity fractions by means of isoelectrofocusing [8]. activity of the L-type isozyme is known to change under dietary and hormonal conditions [3, 13, 31–38].

Isozyme K PK is widely distributed in most adult tissues and is found in all fetal tissues [3, 19, 28, 39]. Like the L isozyme, K type PK exhibits cooperative kinetics with respect to phosphoenolpyruvate [40, 41] and is affected by many allosteric modifiers. Fructose-1, 6-bisphosphate causes the kinetics to shift from sigmoidal to hyperbolic [41]. L-Phenylalanine, L-isoleucine, L-alanine, L-proline and L-tryptophan are also inhibitors of K type PK [27]. The inhibition by L-phenylalanine, L-isoleucine and L-tryptophan is much stronger in the case of K isozyme than with L-type PK [15, 27]. This isozyme appears to exist in two inconvertible forms which are kinetically distinguishable [41].

Isozyme M is found in skeletal muscles, mammalian heart and brain [6, 17, 42–44]. It displays Michaelis–Menten kinetics with respect to phosphoenolpyruvate and is unaffected by fructose-1, 6-bisphosphate [13].

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The abbreviations used in this paper are: PK—pyruvate kinase (ATP: pyruvate phophotransferase, EC 2.7.1.40): AAF—2 acetylaminofluorene (identical with *N*-2-fluorenyl-acetamide).

It has been shown that K and L isozymes are products of two different genes [45]. The molecular differences between M type and the other isozymes suggest strongly that the M isozyme is also the product of a third independent gene. In fetal liver in early stages of development only K isozyme is present [46–47]. In later stages of ontogeny isozyme M appears [21]. These conclusions are, however, controversial and the problem has been poorly investigated. On the day of birth, isozymes K, L and probably M are observed [21, 46]. In hepatocytes of regenerating liver the biosynthesis of isozyme K is highly increased [20]. However, normal isozyme electrophoretic pattern appears after completion of the regenerating process [20].

Neoplastic transformation changes the isozymic pattern of pyruvate kinase. In well differentiated, slowly-growing hepatomas, isozymes K and L have been observed [46, 48, 49]. The activity of isozyme K in this system was remarkably increased in comparison with the levels in normal liver. In several cases electrophoretic resolution revealed three bands of pyruvate kinase [46, 50]. Although changes in the isozymic pattern of PK were the subject of several reports relatively little is known about the behavior of pyruvate kinase isozymes during the process of hepatocarcinogenesis.

The aim of the present investigation was to examine the isozymic pattern of pyruvate kinase during the process of hepatocarcinogenesis induced by 2-acetylaminofluorene (AAF) and to compare these findings with the histological alteration of liver. Because isozymes have been shown to be excellent gene markers it seems possible to elucidate the functioning of pyruvate kinase genes.

As the carcinogen we used 2-acetylaminofluorene because the effects of this compound constitute a useful model for the study of biochemical changes during neoplastic transformation of liver in rats. The lesions develop slowly over a period of months and can be followed from hyperplastic areas to hyperplastic nodules and later to hepatomas.

MATERIALS AND METHODS

Chemicals

Phosphoenolpyruvate (tricyclohexylammonium salt) and NADH were purchased from Sigma (St. Louis, MO, USA), fructose-1, 6-bisphosphate was obtained from Koch-Light Labs. (Colnbrook, Bucks, England). ADP was supplied by the School of Medicine in Łódź (Poland), lactic dehydrogenase was purchased from Boehringer (Ingelheim, W.-Germany) and L-phenylalanine was from Fluka (Switzerland). 2-Acetylaminofluorene was synthetized by Dr. R. Tyka and kindly supplied by the Technical University of Wrocław.

Animals

One hundred and thirty male albino rats (Wistar strain) weighing 150–200 g, of 12 weeks of age, were divided in two groups and fed as described previously [51]. The first group (30 animals) was fed on bread crumbs and water *ad libitum* with carrots one day per week. The animals of the second group (100 rats) were maintained on the same diet supplemented with 11.25 mg AAF per rat daily. The rats of both groups were killed 2, 3, 4, 5, 6, 7, 8, 9 and $9\frac{1}{2}$ months after the diet began. The animals were decapitated between 8 and 9 a.m. After exsanguination, livers were quickly removed, washed with cold 0.01 M Tris–Cl–1mM EDTA buffer, pH 7.5. Each liver was sectioned into small pieces and these were randomly selected for histological examination and biochemical analysis. At the end of experiment nine animals with liver tumors were left. Homogenization of liver tissues was carried out with a motor-driven homogenizer with teflon pestle in 1:0.5 w/v of 0.01 M Tris–Cl-1 mM EDTA buffer, pH 7.5. The homogenates were centrifuged at 75,000 × *g* for 1 hr at 3°C. The clear supernatants were used for assay of PK-activity and electrophoresis.

Cellulose acetate electrophoresis

Zone electrophoresis on cellulose polyacetate strips (Sepraphore III, Gelman Instr., Co., Ann Arbor, Michigan, USA) was performed following the method described by Susor and Rutter [4]. Pyruvate kinase isozymes were revealed by the procedure described by the above authors [4].

Enzyme assay

Activity of pyruvate kinase was determined spectrophotometrically following the decrease of absorbance of NADH at 340 nm by the method of Bücher and Pfleiderer [42], as modified recently by Osterman and Fritz [3]. The reaction was started by the addition of 1 µl of liver extract to a 3-ml cuvette containing the following chemicals (final concentration): 50 mM Tris–Cl, pH 7.5, 1 mM EDTA, 75 mM KCl, 8 mM $MgSO_4$, 2 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH and 1 U/ml of lactic dehydrogenase. The activity measurements of PK in the presence of L-phenylalanine were carried out in the same way but in the presence of 0.3 or 2 mM phosphoenolpyruvate and 0.6 or 2 mM of L-phenylalanine,

respectively. All assays were performed at 25°C using either an Acta M VI (Beckman Instr., Fullerton, USA) or Specord UV-VIS (Zeiss-Jena, E. Germany) spectrophotometers.

RESULTS

Zone electrophoresis of pyruvate kinase in liver extracts is shown in Fig. 1. Corresponding photomicrographs of rat liver sections obtained during hepatocarcinogenesis are presented in Fig. 2. The PK isozyme pattern of rats from the control group remained unchanged. The activity which corresponds to the isozyme L is very strong and only a trace of activity of isozyme K could be detected (Fig. 1A). The specific activity of PK was in the range 0.84–1.60 U/mg protein (Table 1).

In early stages of hepatocarcinogenesis, after 2–4 months of AAF administration when only slight pleomorphism of hepatocytes was found (Fig. 2B) the specific activity of PK was slightly decreased (Table 1). However, electrophoretic examination revealed an increase of activity of isozyme K (Fig. 1B).

After 5–7 months of administration of AAF, when microscopic examination revealed cirrhosis of the liver, focal dilatation and proliferation of bile ducts (Fig. 2C), marked decrease of specific PK activity was observed (Table 1). In the electrophoretic profile the activity of isozyme L was diminished in comparison to the control group (Fig. 1C).

After feeding AAF for 8–9 months the rats livers contained two types of tumors: hepatoma and cholangiocarcinoma (Fig. 2D and E). Biochemical examinations were performed in six hepatomas and three cholangiocarcinomas. The specific activities of PK in extracts of the three cholangiocarcinomas lie in the range 0.50–0.78 U/mg protein (Table 1). Electrophoresis demonstrated three activity bands of PK (Fig. 1D). Two of them corresponded to isozymes L and K, the third very faint band had the same mobility as isozyme M. This was proved by performing electrophoresis with muscle extract.

The activity of PK in hepatomas are summarized in Table 1. In two tumors the activities were within the range of control values. In two cases the specific activities were approximately 40% of the control values; in two hepatomas the PK activities per mg of protein were very low. The electrophoretic profile of three hepatomas displayed the presence of two strong activity bands of both isozymes K and L (Fig. 1E). In two liver tumors electrophoresis demonstrated three activity bands L, K and probably M (Fig. 1G). In one hepatoma only isozyme K was present (Fig. 1F).

To support the assumption that the activity band with cathodic mobility observed in hepatomas is derived from isozyme K, measurements of PK activity were performed in the presence of L-phenylalanine, a strong inhibitor of K isozyme. The results are given in Table 2. The PK activity in normal liver was only slightly diminished in contrast to the hepatoma (Fig. 1F), where more than 90% of activity was inhibited.

DISCUSSION

The above data give the evidence for an increase of biosynthesis of isozyme K during hepatocarcinogenesis caused by AAF administration. It is interesting that after 2–4 months of drug administration when only pleomorphism of hepatocytes was observed, the increased biosynthesis of K isozyme takes place.

In the precancerous state, i.e. proliferation of hepatocytes and bile ducts with concomitant cirrhosis, further increase of activity of isozyme K is observed. Simultaneously the specific activity of pyruvate kinase decreased to 60% of the control values. These results are similar to the data reported by Walker and Potter [46], who induced hepatomas by 3'-methyl-4-diaminoazobenzene. The above authors observed elevated activity of isozyme K and decreased total PK activity. As in our experiment these investigators took pieces of cancerous liver for biochemical and histological examinations. However, Yanagi *et al.* [52], who also used AAF as a carcinogenic agent and isolated hyperplastic nodules, observed a twofold increase of K-type PK but without change in total PK activity. In non-nodular areas neither changes in isozymic pattern nor in total activity were found [52]. Perhaps these different results were caused also by different ways of administration of carcinogens.

AAF besides its carcinogenic properties is also toxic [53]. Cirrhosis, observed in the precancerous state is caused by its toxic properties. The question is raised whether cirrhosis itself caused changes in isozymic pattern of PK. However, we have not observed any alteration in the isozyme pattern of PK after prolonged administration of carbon tetrachloride when rat liver cirrhosis is evident [54]. Thus changes in the isozymic pattern seem to be characteristic for neoplastic transformation.

For three cholangiocarcinomas specific PK activity was about 40% lower than control values. For the same tumour Yanagi *et al.* [52], obtained very high total PK activity, similar to the poorly differentiated hepatomas. Our results

*Table 1. Histological findings, total enzymatic activity and electrophoretic pattern of isozymes of pyruvate kinase during hepatocarcinogenesis inducted by AAF in rat liver**

Time after beginning of administration of AAF in months	Number of animals	Histological findings	Specific enzymatic activity†			Isozymic pattern observed on electrophorogram‖		
			Range	Mean ± S.D.‡	P§	Isozyme L	Isozyme K	Isozyme M
Control group	18	Normal, health liver	0.84–1.60	1.13 ± 0.22	—	+ + +	+	
2–4	7	Slight pleomorphism of hepatocytes	0.52–1.25	0.96 ± 0.27	<0.2	+ + +	+ +	
5–7	6	Proliferation of hepatocytes and dilatation and proliferation of bile ducts	0.43–0.80	0.61 ± 0.14	<0.001	+ +	+ +	
8–9	3	Cholangiocarcinoma	0.50–0.78	0.64 ± 0.14	<0.002	trace	+ + +	trace
8–9	1	Hepatoma		0.98		+	+ + +	
	1	Hepatoma		0.84		+	+ + +	+
	1	Hepatoma		0.47		+	+ + +	+ + +
	1	Hepatoma		0.41		+ +	+ +	
	1	Hepatoma		0.27		+	+ + +	
	1	Hepatoma		0.18		+	+ + +	

*The way of feeding animals, methods of detection of pyruvate kinase isozymes, conditions of electrophoresis and determination of pyruvate kinase activity are given in "Materials and Methods" section.

†μmole of phophoenolpyruvate used per 1 min per 1 mg of protein at 25°C.

‡ Mean value of pyruvate kinase activity ± standard deviation.

§*P* value for the difference of mean enzymatic activity between each group and control group as determined by Student's *t*-test.

‖Visual determination of + + + high, + + medium and + low activity of given isozyme.

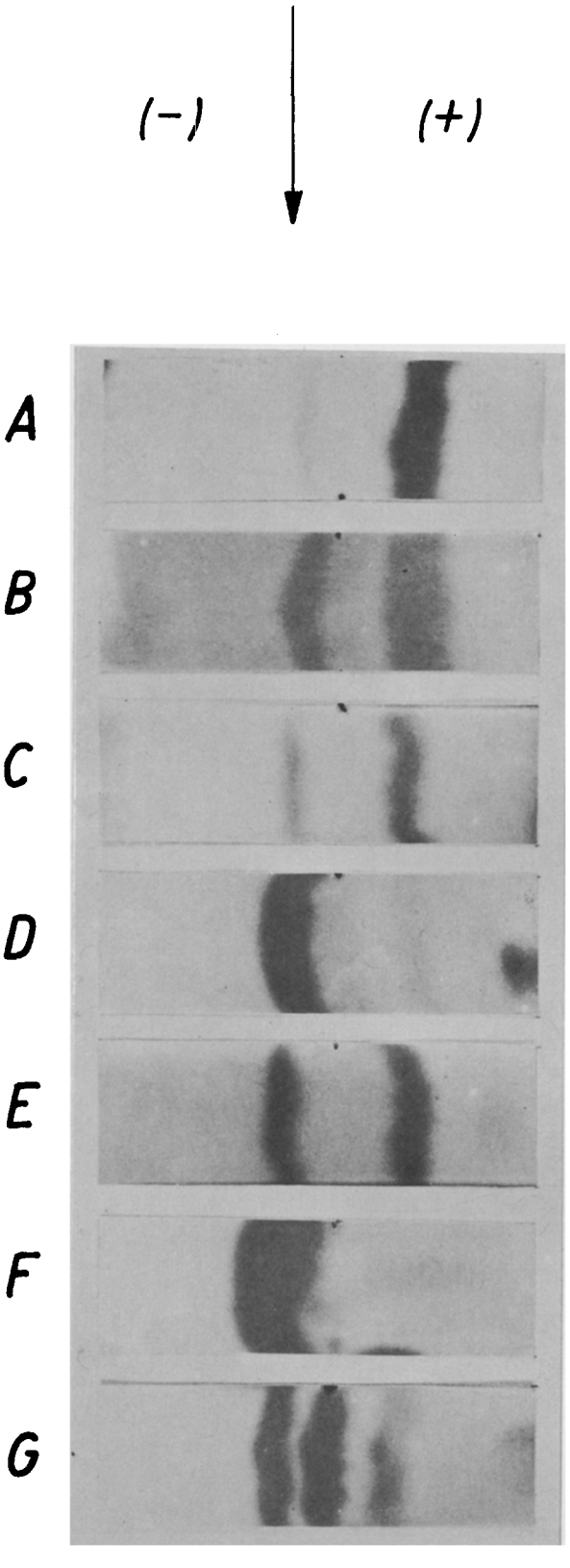

Fig. 1. Zone electrophoresis of PK in extracts of liver from rats during hepatocarcinogenesis induced by AAF. Procedures for tissue homogenization, electrophoresis and method of detection of isozymes are described in "Materials and Methods". Small dark dots on the electrophorograms and the arrow indicate the origin.

A. PK isozyme pattern in liver extract of rat from control group. The anodic band represents isozyme L. The trace of cathodic activity band is isozyme K.

B. The pattern of PK isozymes after 2 months of AAF administration.

C. PK isozyme pattern in liver extract after 6 months of AAF administration.

D. PK isozymes pattern in extract of cholangiocarcinoma obtained after 8 months of administration of AAF. The third, very faint activity band occupies a position near starting point.

E, F and G. PK isozyme patterns in extracts of hepatomas obtained during hepatocarcinogenesis.

E. Two isozymes K and L of approximately equal intensity: 9 months of AAF administration.

F. Only K isozyme can be detected: 9 months of administration of carcinogen.

G. Three activity bands of PK: $9\frac{1}{2}$ months of administration of AAF.

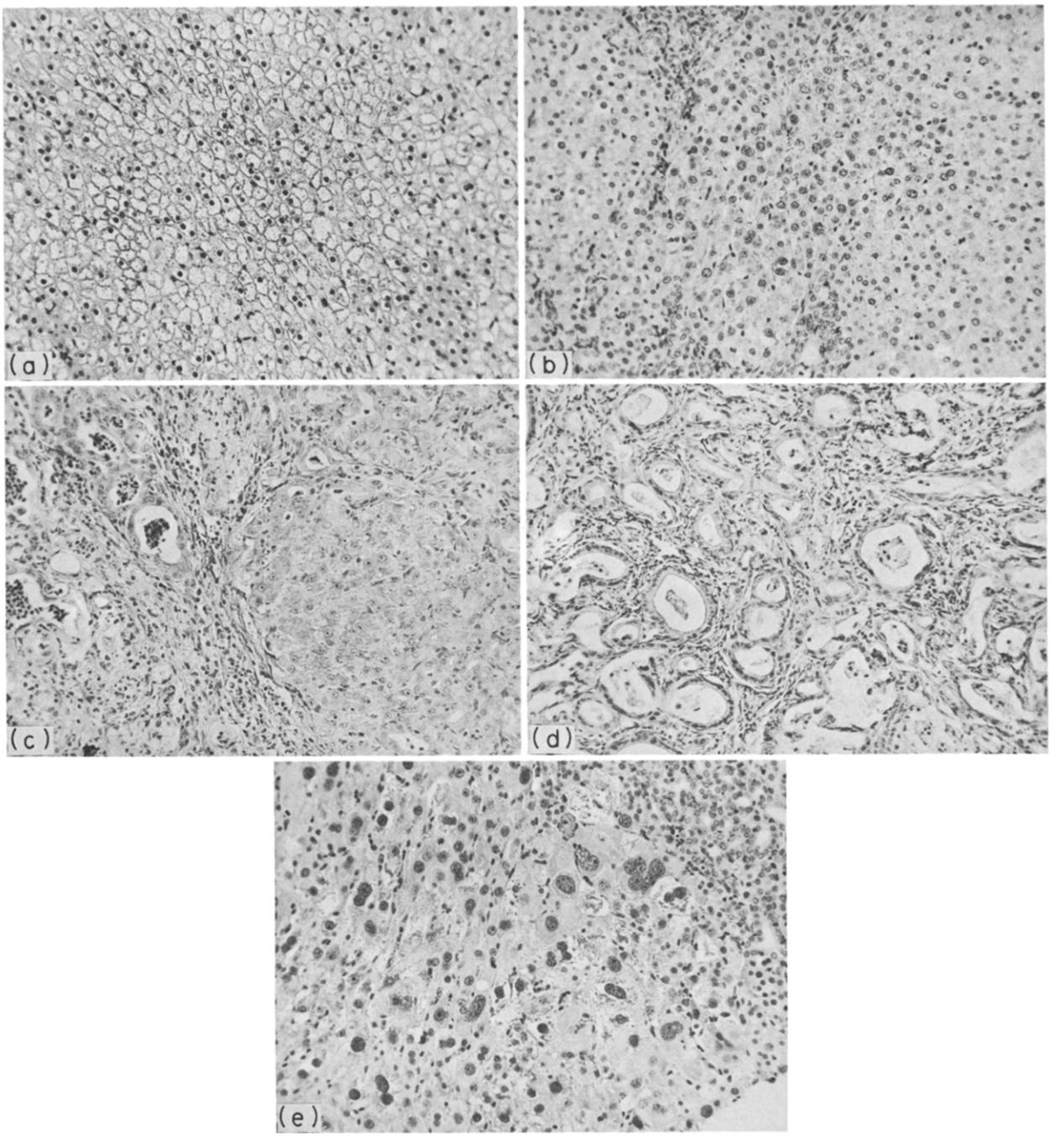

Fig. 2. Photomicrographs of sections of rat livers during hepatocarcinogenesis induced by AAF.

A. Normal liver in control group. H and E, × 130.

B. Section of rat liver prepared after 2 *months of administration of AAF; slight pleomorphism of hepatocytes. H and E,* × 130.

C. Rat liver after 6 *months of administration of AAF; proliferation of hepatocytes and dilatation and proliferations of bile ducts, H and E,* × 130.

D. Section of rat liver after 8 *months of administration of AAF; cholangiocarcinoma. H and E,* × 130.

E. Section of rat liver after 9 *months of administration of AAF; hepatoma. H and E,* × 130.

Table 2. Inhibition of pyruvate kinase activity in normal rat liver and in hepatoma by L-phenylalanine

Concentration of L-phenylalanine and phosphoenolpyruvate*		Remaining activity	
		Normal liver	Hepatoma
L-Phenylalanine Phosphoenolpyruvate	=2 mM =2 mM	81.4%	5.0%
L-Phenylalanine Phosphoenolpyruvate	=0.6 mM =0.63 mM	82.6%	10.0%

*Concentrations of all remaining reagent used for pyruvate kinase assay are given in "Materials and Methods" section.

†Pyruvate kinase activity remaining under the conditions of analysis expressed as percentage of the activity of identical sample but assayed without L-phenylalanine.

of PK activity measurements and electrophoretic pattern for six well-differentiated and slowly growing hepatomas are in agreement with the data reported for well differentiated Morris hepatomas [48, 52, 55].

The electrophoretic profile of five tumors revealed the presence of three activity bands. Two of them corresponded to isozyme K and L while the third activity band can be ascribed to either isozyme M or to a particular hybrid of isozymes L and K, since one of the L–K hybrid set should have a mobility similar to isozyme M. The presence of this molecular form of PK in hepatomas was also observed by Walker and Potter [46] as well as Tanaka *et al.* [50]. *In vitro* the L–K hybrid set was obtained by Dyson and Cardenas [56]. Susor and Rutter [57] and Susor *et al.* [58], electrophoretically resolved 5-membered set of PK-activity bands in extracts of 18-day fetal liver and in the livers of newborn rats. They ascribed the observed electrophoretic pattern to the 5-membered L–K hybrid set. However, Criss [9] using electrofocusing technique found in well differentiated hepatomas four molecular forms of PK one of which has an isoelectric point identical to isozyme M. During hybridization *in vitro* no preferential formations of only one hybrid was observed [56, 57]. Thus, the hypothesis that the third molecular form of PK is the isozyme M seems to be rationalized. We want to stress that the electrophoretic technique on polyacetate strips is sufficiently sensitive to resolve PK hybrids, because in control experiments we revealed a 5-membered hybrid set in rat jejunum extracts, according to Osterman and Fritz [3].

The above results may lead to the following conclusions concerning the expression of PK genes in hepatocytes during hepatocarcinogenesis. In adult precancerous liver as well as in liver tumors only L and K isozymes were detected. The absence of L–K hybrids suggests that in a typical hepatocyte two PK genes are not functioning at the same time. Thus the synthesis of L isozyme stops when the synthesis of K isozyme begins in the same hepatocyte. Garnett *et al.* [20] reported that the L and K genes behave in a similar manner in regenerating rat liver. The expression of pyruvate kinase genes is, therefore "alternate" and similar to such enzymes as: phosphofructokinase [50, 59], esterase [60], hexokinase (Weinhouse *et al.*, 1972), glycogen phosphorylase [61], adenylate kinase [55] and branched chain amino acid transaminase [62]. However, the functioning of PK genes during neoplastic transformation is quite different in comparison with aldolase for which simultaneous expression of A and B genes in the same hepatocyte was observed [63].

During the hepatocarcinogenesis the switching on of the gene for K isozyme of PK is associated with switching off the gene for L isozyme. In some hepatomas presumably the third PK gene is switched on. Therefore, the isozymic pattern is similar to that of fetal liver. This is consistent with the hypothesis of dedifferentiation during neoplastic transformation [64–66].

The biochemical changes in gene expression of PK are characteristic for carcinogenesis and even in early stages of oncogeny can be distinguished from cirrhosis. The changes are of great importance to liver metabolism. The PK is a rate-limiting glycolytic enzyme. In gluconeogenic tissues (e.g. liver) the predominant form of PK is L isozyme, which is especially sensitive to changes in concentration of various metabolites. The compounds which are utilized for glucose synthesis (e.g. L-alanine, free fatty

acids, acetyl-CoA) inhibit the activity of isozyme L [23, 67, 68]. The activity of isozyme K is not so precisely controlled [32, 33, 69]. Therefore, the elevated biosynthesis of isozyme K in rat liver promote glycolysis which reflects the growing demand in this tissue for energy and carbon sources for the new tumor-cell formation.

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Cell Cycle Stage-Dependent Induction of G_2 Phase Arrest by Different Antitumor Agents*

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Abstract—*A variety of chemically unrelated antitumour compounds has been shown to block cell cycle progression in G_2 phase. Using thymidine synchronized human lymphoid cells, we have investigated the cycle stage dependency of this kinetic response. For anthracycline antibiotics (adriamycin, adriamycin–DNA complex and rubidazone) and the epipodophyllotoxin derivative VP16, cells in S phase were most susceptible to the induction of G_2 block in the immediate life span. Alkylating agents (melphalan, peptichemio and Yoshi 864) displayed a peculiar sensitivity pattern: G_1 cells were effectively delayed in the subsequent G_2 phase, whereas this kinetic event was transmitted to the daughter cell population when treatment was commenced in G_2 phase. The time of manifestation of G_2 block after incubation in S phase varied among the three alkylating agents and was a function of the position in S phase. In general, there was no correlation between phase sensitivity for induction of G_2 block and lethal cell damage.*

INTRODUCTION

A VARIETY of physical and chemical agents have been reported to interfere with cycle progression in the G_2 phase of mammalian cells [1–11]. In fact, Tobey recently showed that some of these agents can arrest cells at distinct stages of G_2 phase [12]. In previous publications, we demonstrated that the extent and duration of antitumor drug-induced G_2 block are a function of the concentration and time exposure to the agent [13–17]. In this report, we provide evidence that the position of cells in the cell cycle at the time of drug treatment is another determinant for onset, degree and duration of the G_2 arrest.

MATERIALS AND METHODS

Our experiments were carried out on a human lymphoma cell line (T_1 cells), which was derived from a patient with lymphocytic lymphoma [18]. T_1 cells are grown as monolayer cultures in Ham's F10 medium supplemented with 20% fetal calf serum, glutamine, vitamins and antibiotics, in a 5% CO_2 humidified atmosphere at 37°C. Employing the pulse labeled mitoses technique, the recently determined cell cycle time was 31 hr with $T_{G_1} = 15$ hr, $T_S = 10$ hr, $T_{G_2} = 6$ hr [16]. The influence of cell age at the time of drug application on magnitude and duration of subsequent G_2 block was analyzed on synchronized cultures. Previous studies had demonstrated that excess thymidine treatment yields a high degree of cell synchrony maintained over the period of at least 1 cell cycle [19, 20]. At selected intervals after release of the thymidine block, T_1 cells were treated for 1 hr with the antitumor agent under investigation at a concentration that had proven effective in inducing G_2 phase arrest after exposure of asynchronous cells. Cycle progression was monitored by serial measurements of cellular DNA content after fluorochromation with mithramycin [21] in a Phywe ICP 11 pulse cytophotometer [20, 22]. DNA histograms were obtained at 6–12 hr intervals for a total period of at least 6 days (i.e. 4–5 times the generation time of exponentially growing cultures). Thus, the time of onset, degree and duration of delay in the transit through G_2 phase could be determined as a function of cycle stage where the treatment was conducted.

Three anthracyclines, 3 alkylating agents, and

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Table 1.

Drug	Drug abbreviation	Concentration μg/ml
Anthracycline antibiotics		
Adriamycin; hydroxydaunorubicin	ADR	0.5
Adriamycin–DNA complex (herring sperm-DNA:ADR; 20:1)	ADR–DNA	0.5 (ADR)
Rubidazone; benzoylhydrazone daunorubicin	RUB	1.0
Alkylating agents		
L-Phenylalanine mustard	L-PAM	2.5
Peptichemio; multipeptide complex of *m*-(di-(2-chloroethyl)-amino) L-Phenylalanine	PEP	5.0
Yoshi 864; 3,3′-iminodi-1-propanol, dimethanesulfonate (ester), hydrochloride	Yoshi	100
VP-16-213: 4′demethylepipodophyllotoxin 9-(4,6-0-ethylidene-β-D-glucopyranoside)	VP-16	5.0 10.0

the epipodophyllotoxin derivate VP16, were investigated at the concentrations indicated in Table 1. For the concentrations and incubation times used in this analysis, there was no significant interference with the transverse through stages of the cycle other than G_2 phase [11, 13–17].

All drug solutions were prepared with growth medium immediately prior to each experiment. ADR (in vials of 10 mg) was obtained from Farmitalia, Milan, Italy. ADR–DNA complex was prepared as follows: herring sperm DNA (highly polymerized, type VII; Sigma Chemical Company, St. Louis, MO), at a concentration of 2.34 mg/ml in 0.9% NaCl solution, was kindly provided by Dr. A. Trouet, Laboratoire de Chimie Physiologique, Université de Louvain, Louvain, Belgium. The DNA solution was autoclaved for 15 min at 120°C and cooled slowly. One-tenth of a milliliter of a 10 mg/ml aqueous solution of ADR was dissolved in 5 ml of DNA solution. Further dilution was performed with 0.9% NaCl solution and medium, respectively, for the appropriate concentration of ADR, i.e. 0.5 μg/ml. Thus, a 20:1 molar ratio of DNA mononucleotide to ADR was achieved [23]. RUB was supplied by the Societé des Usines Chimiques Rhône-Poulenc. VP16-213, manufactured by Sandoz Laboratories, Basel, Switzerland, was received in an organic solvent and further dilutions were made in growth medium. Yoshi 864 was supplied in 100 mg vials by Ben Venue Laboratories, Bedford, Ohio and reconstituted in 5 ml of distilled water. L-Phenylalanine mustard was obtained as Alkeran through Burroughs Wellcome Company, Research Triangle Park, N.C. One hundred milligrams of L-PAM was dissolved in 1 mg of Wellcome acid alcohol, and 9 ml of Wellcome diluent was added. Further dilutions were made with growth medium. PEP, a multipeptide complex of *m* - (di - (2 - chloroethyl) - amino) - L - phenylalanine was kindly provided by Professor A. De Barbieri, Istituto Sieroterapico Milanese S. Belfanti, Milan, Italy.

RESULTS

ADR irreversibly accumulated more than 70% of the cell population in G_2 phase, independent of cycle stage of administration (Fig. 1A). However, while drug exposure in S and G_2 phase elicited an immediate accumulation, treatment in G_1 phase yielded a progressive G_2 increment over a period of 4 days. This effect suggests that some cells treated before the onset of DNA synthesis can escape the blocking effect of ADR during their life span. However, their progeny will be blocked in the subsequent G_2 phase, thus contributing to the total accumulation in that stage. When ADR was administered complexed to DNA, a phase dependent sensitivity in G_2 arrest induction was observed (Fig. 1B); treatment of cells throughout S and in early G_2 phase elicited a largely irreversible G_2 block of 80–90%; in contrast, treatment in late G_2 and in G_1 phase caused a transient increment of cells in G_2 (20 and 40%, respectively). The degree and extent of the G_2 block following incubation with RUB was also a function of cell age; treatment of T_1 cells in early and mid S phase was most effective in interfering with the traverse through the subsequent G_2 phase (Fig. 1C).

Exposure of T_1 cells to three alkylating agents showed a novel feature of the kinetic response pattern; the time of manifestation of the delay in G_2, whether evident during the life span of the treated cells or within that of their immediate progeny, varied with cell age at the time of drug exposure (Fig. 2). Treatment of T_1 cells with L-PAM in G_1 phase effected a largely irreversible G_2 increment of 75% within the same life cycle (Fig. 2A). Drug incubation in S and G_2 phase did not interfere with cycle traverse to mitosis

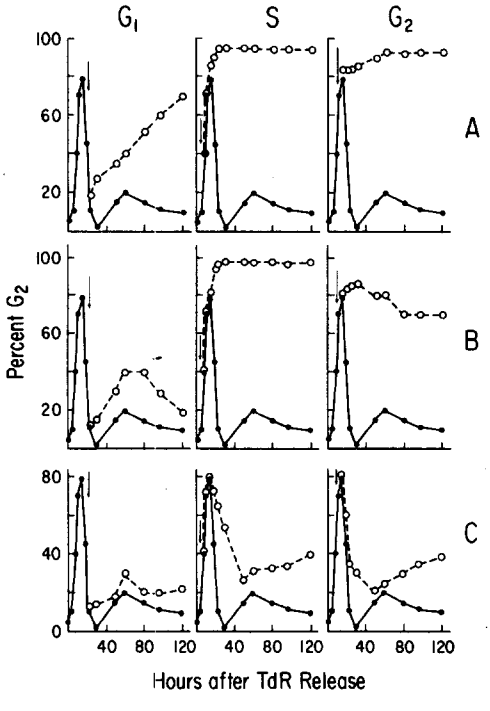

Fig. 1. Effect of anthracycline antibiotics on G_2 traverse of T_1 cells as a function of the stage of treatment (G_1, mid S and early G_2 phase). The solid lines (●—●) represent the proportion of control cells in G_2 phase, the dashed lines (○ - - - ○) connect values after 1 hr treatment in the 3 different phases. The time of treatment is indicated by the arrows. A: ADR 0.5 μg/ml, 1 hr. Induction of a high degree of irreversible G_2 accumulation of 70–95%, independent of the cycle stage of drug exposure. The time of G_2 maximum is delayed for cells treated in G_1 phase. B: ADR–DNA 0.5 μg/ml, 1 hr. Compared to ADR, initiation of treatment in G_1 phase causes only a transient G_2 accumulation of 40%. C: RUB 1.0 μg/ml, 1 hr. S phase cells are most effectively delayed during their traverse through G_2 phase, while exposure of cells in G_1 phase effects a minor increase of the G_2 compartment.

and the subsequent progression through G_1 and S phase of the progeny cells; yet, a significant block was noted in the consecutive G_2 phase. Thus, in all circumstances, DNA synthesis in drug free medium preceded the induction of G_2 block by L-PAM. PEP mimicked very closely the kinetic effects of L-PAM in asynchronous cultures; for a given drug concentration, similar degrees of G_2 accumulations were observed. Yet, notable differences were noted on synchronized cells. Cells treated with PEP in both G_1 and early and mid S phase were effectively delayed upon their arrival in the immediate G_2 phase (Fig. 2B). Drug exposure of cells in late S and G_2 phase transmitted this perturbation effect to the daughter cells. For Yoshi 864 (Fig. 2C), similar, yet more transient cycle stage dependent manifestations of G_2 delay were noted.

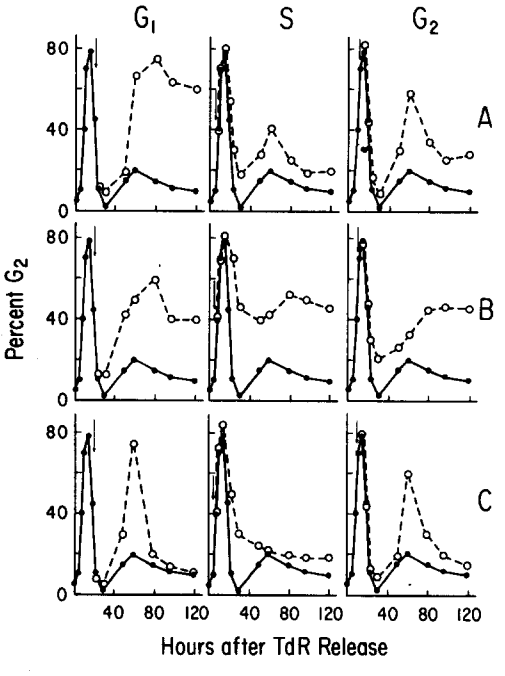

Fig. 2. Effect of alkylating agents on G_2 traverse of T_1 cells as a function of the stage of treatment (G_1, mid S and early G_2 phase). Symbols as in Fig. 1. The time of treatment is indicated by the arrows. A: L-PAM 2.5 μg/ml, 1 hr. Induction of marked accumulation in the immediate G_2 phase following treatment of cells in G_1. Exposure of cells in S and G_2 phase does not impair the traverse through the immediate G_2 phase nor the progression through G_1 and S of the progeny cells. G_2 transit of the daughter cells is prolonged, visualized by the G_2 increase at 60 hr. B: PEP 5.0 μg/ml, 1 hr. Treatment of cells in G_1 phase elicits a high degree of G_2 accumulation in the target cells. Following exposure of cells in early and mid S phase, a delay of G_2 transit is noted and 40–50% of the total population is irreversibly arrested in G_2 phase. Cells treated in late S and G_2 phase are blocked in the G_2 phase of the next life cycle. C: Yoshi 864 100 μg/ml, 1 hr. Manifestation of G_2 accumulation as a function of cycle stage is similar to that observed after treatment with PEP. However, peal values of G_2 increase are higher, and the effect is largely reversible.

Induction of G_2 accumulation by VP16 was also age dependent (Fig. 3); for the lower concentration of 5 μg/ml (Fig. 3A), sensitivity to the induction of a G_2 block decreased in the following order: early and mid S phase > later S phase > early G_2 > late G_2 > G_1 phase. Dose escalation to 10 μg/ml (Fig. 3B) partially abrogated the pronounced phase sensitivity: treatment in both early and late G_2 phase was equally effective as drug exposure in S phase to produce maximal G_2 accumulation.

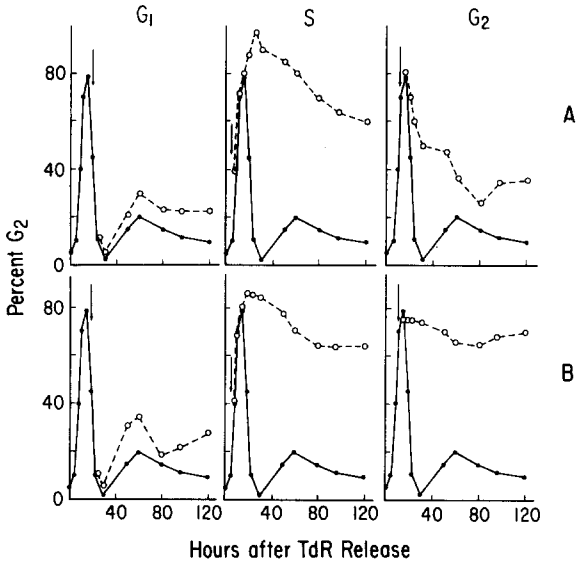

Fig. 3. Effect of VP16 (Symbols as in Fig. 1) on G_2 traverse of T_1 cells as a function of the stage of treatment (G_1, mid S and early G_2 phase). The time of treatment is indicated by the arrows. The cell age dependent induction of G_2 accumulation is modulated by drug concentration. A: 5 μg/ml, 1 hr. Treatment in mid S phase produces the highest degree and longest duration of G_2 accumulation. Following exposure of cells in early G_2 phase, about 30% of the total population experienced only a minor delay in G_2 phase; the residual population resumes cycle progression with a delay of 35 hr. Treatment of G_1 cells causes a small G_2 increment of 10%. B: 10 μg/ml, 1 hr. Dose escalation renders G_2 cells equally susceptible to the induction of G_2 block as S phase cells.

DISCUSSION

Our results indicate that a variety of chemically related and unrelated antitumor compounds delay cycle progression in G_2 phase. However, the phases of greatest sensitivity for induction of this shared kinetic response differ. For alkylating agents, the significance of cell age as a determinant for this kinetic perturbation was particularly striking, since it conditioned the time of manifestation of a G_2 block in the life span of the treated cells or in their immediate progeny.

Previous investigations using this cell line have shown that there is no correlation between the degree of reduction in survival and the extent or duration of G_2 block [15, 16, 17, 24, 25]. Likewise, there is a discrepancy between phase sensitivities for lethal damage and degree or duration of G_2 accumulation [26]. Only for L-PAM and Yoshi 864, maximal cell kill resulted from treatment in those phases where a G_2 block was induced in the same life cycle. Thus, kinetic and lethal effects may not be related and caused by injuries to different target sites.

Our studies demonstrate that various antitumor agents elicit perturbation of normal traverse of mammalian cells through the cell cycle. Although this effect has a common endpoint, G_2 phase arrest, different drugs yield this result via different modes or pathways. This is attested by the variation in cell cycle stage where maximum effect can be elicited after brief treatment. The shared effect of G_2 phase arrest may be the end result of damage or inhibition of certain postulated division-specific enzymes [27], while cell cycle dependency of the various drugs may be the result of damage or inhibition of precursors of these specific enzymes. For VP16, we have demonstrated that the cell age dependency of G_2 phase arrest can be modulated by drug concentration. Thus, treatment with such drugs may provide a valuable probe for investigating the nature of the macromolecular mechanisms related to cell division.

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Density-Dependent Mitosis in the Walker 256 Carcinoma and the Influence of Host Age on Growth

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Abstract—*The Walker 256 carcinoma was tested for the density dependence of cell division that has been found in normal tissue. It was found that autoregulation did occur to some extent in the tumours growing in young hosts, but that, in old hosts, the mitotic pattern resembled that observed in normal tissues. The rates of tumour growth and overall cellular proliferation were also significantly lower in old compared with young hosts.*

INTRODUCTION

DESPITE early theories [1–3] that neoplasia is a result of uncontrolled mitotic regulation there is increasing evidence that a growth regulatory system persists in certain tumours. This work has been mainly concerned with the interaction between primary tumours and secondary tumour cell population, that arise elsewhere in the body, and has, therefore, centred around hypothetical systemic factors [4–7].

Where interest has concentrated on growth control within solid tumours, the aim has been to investigate the retardation of growth as the tumours increase in size. This growth retardation has been attributed to a relative inadequacy of local blood supply [8], competition for nutrition [8, 9], inhibitory tumour by-products [10] and immunological factors [11]. Relatively little attention has been paid to the possibility that the phenomenon is an expression of an autoregulatory system similar to that governing the size of mitotic cell populations in normal organs. It was the aim of the present work to test Walker carcinoma for the density dependence of cell division that has been found in normal thymus [12], and secondly to examine the role of the host in growth regulation.

MATERIALS AND METHODS

Animals

Twenty or 120 week old male rats of an inbred WAB substrain, were used throughout the following experiments. Tumour tissue was obtained from 20 week old donor rats bearing a solid, subcutaneous Walker 256 carcinoma. A 6 mg fragment of tumour tissue was inserted subcutaneously into the middorsal region. The animals were killed, by cervical dislocation, 3, 4, 6, 7, 8, 9 or 10 days later and the tumour tissue removed.

Colchicine administration

Mitosis was hindered at metaphase by intraperitoneal administration of 0.2 mg colchicine per 100 g body weight, 4 hr prior to death.

Histology

Tumour tissue was fixed in Bouin's fluid for 36 hr, dehydrated through graded alcohols and embedded in paraffin wax. Sections were cut at 5μm and stained with Mayer's haematoxylin.

Cell counts

The total cell fragment numbers in 200 microscope areas (180 μm dia) were counted and the number of mitotic figures scored. Necrotic areas were avoided and only obvious tumour cells counted. The actual whole cell numbers were obtained by application of the Floderus correction formula [13].

RESULTS

Figure 1 shows the growth of the tumours in 20 week- as compared with 120 week-old animals. It can be seen that during the initial stages, when the tumour is adapting to its new environment, little growth occurred and no difference in the tumour size was observed between the two age groups until

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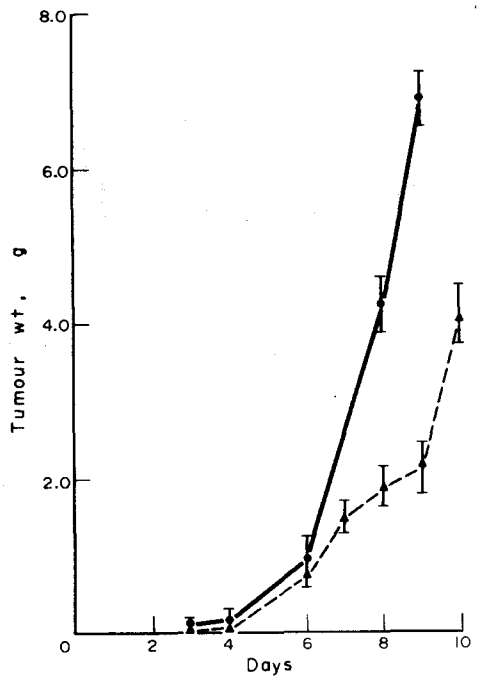

Fig. 1. The growth rate of subcutaneous Walker 256 carcinoma in young ● and old ▲ hosts, from 0 to 10 days after implantation. (Results expressed as mean ± S.E. tumour weight g n = 10.)

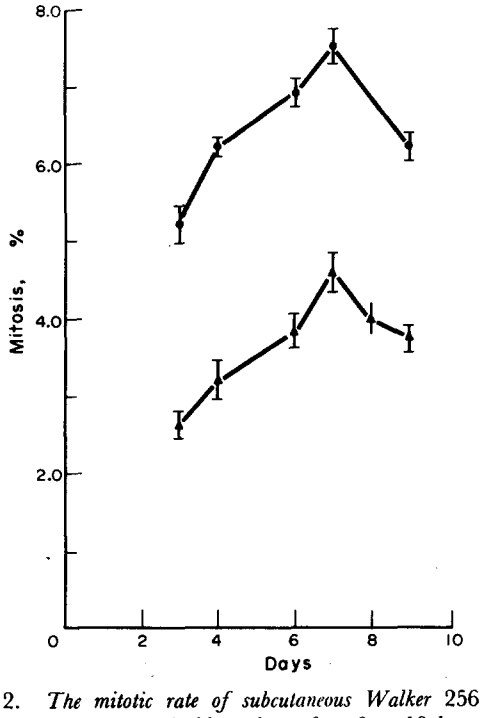

Fig. 2. The mitotic rate of subcutaneous Walker 256 carcinoma in young ● and old ▲ hosts from 0 to 10 days after implantation. (Results expressed as mean ± S.E.% mitosis.)

six days after transplantation. Thereafter, the growth rate of the tumours in the older group was significantly lower than that observed in 20 week-old animals.

The tumour growth curve in the younger hosts conformed to the Gompertzian model of Walker 256 tumour elaborated by Laird [14], but the tumour growth retardation in the older animals resulted in an almost complete deviation from this pattern.

In accordance with the growth pattern, overall cellular proliferation in the tumours in 120 week-old animals was significantly lower than that observed in 20 week-old hosts (Fig. 2), at each stage of the tumour growth. The magnitude of this difference in tumour cellular proliferation, between the two age groups, increased from 100% 3 days after transplantation to 300% 6 days post-operatively. It can be seen, therefore, that a significant difference in tumour cell proliferation between the two groups occurs before any difference in the tumour weight becomes apparent.

The pattern of overall tumour cell proliferation for the two groups was identical even though the absolute values were different. In both age groups, cellular proliferation increased up to a peak at 7 days after transplantation and thereafter declined. The rapid rate of cellular proliferation observed between 3 and 7 days after transplantation corresponded to the period of high growth rate when the newly formed cells outnumber those lost due to degeneration. Tumour growth continued despite the decline in mitotic rate after 7 days. However, at this time, the tumours in both groups of animals underwent extensive necrosis and liquefaction, and the hosts became moribund.

In order to investigate the distribution of the mitotic figures in the tumours the microscope areas counted were categorized according to the cell density. Category 1 consisted of between 1 and 10 cells per area, through to category 8 with between 71 and 80 cells per area. The distribution pattern of cell density categories during the phase of high proliferation, at 4 days and at the time when mitotic rate was declining at 9 days, is shown in Fig. 3. It can be seen that in both age groups, and at both stages after tranplantation, the cell density distribution patterns were essentially similar. The majority of areas counted fell into categories 5–7, with a slightly wider spread of cell density distribution at 4 days as compared with 9 days. When the rates of mitoses were related to the cell density categories, a definite pattern of mitotic cell distribution emerged (Figs. 4 and 5). During the establishment of the tumour and initiation of a high rate of cellular proliferation at 3 days, mitotic rate was distributed throughout the cell density categories. Following this stage few mitotic figures were observed below

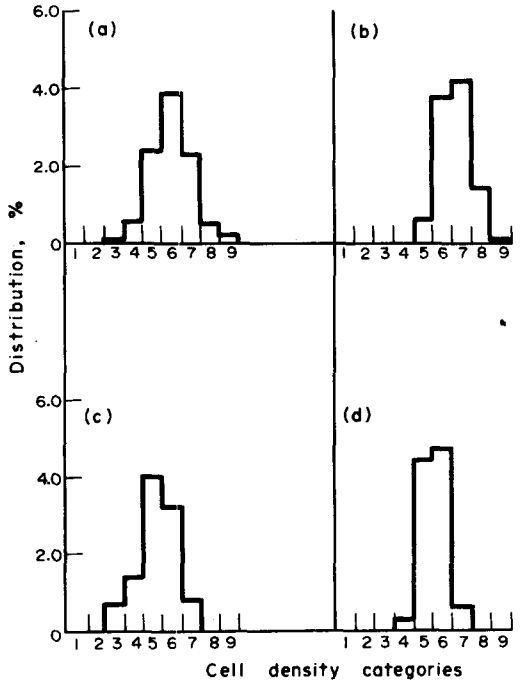

Fig. 3. The distribution of standard microscope areas counted in relation to cell density categories in: the old host A—4 days *and B*—9 days *after implantation and in the young host C*—4 days *and D*—9 days *after implantation (Category 1 consisted of 1–11 cells/area—category 9, 81–90 cells. Results expressed as % distribution of areas).*

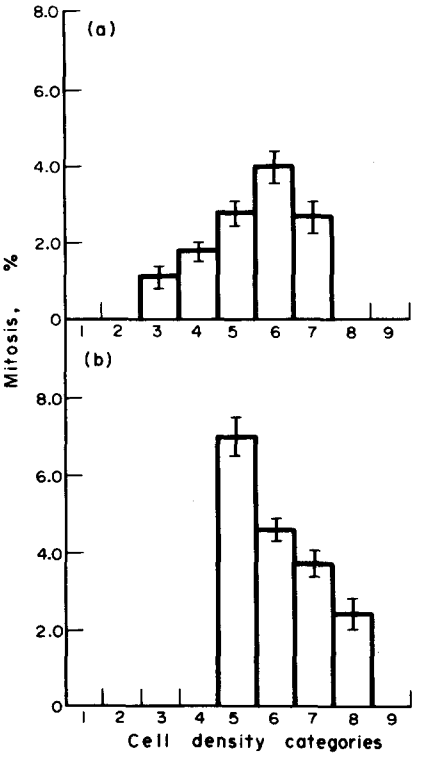

Fig. 5. The mitotic rate of tumour cells, in relation to cell density, in old hosts. A—3 days *and B*—7 days *after implantation. (Results expressed as mean ± S.E.% mitosis for each category.)*

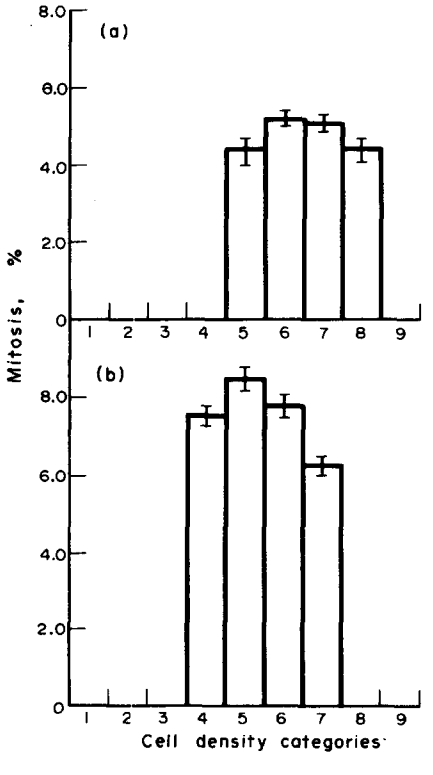

Fig. 4. The mitotic rate of tumour cells, in relation to cell density, in young hosts. A—3 days *and B*—7 days *after implantation. (Results expressed as mean ± S.E.% mitosis for each category.)*

category 5; beyond this category, mitotic rate declined with increasing cell density.

This concept of decreasing mitotic rate with increasing cell density was borne out in tumours established in old hosts and did not fluctuate appreciably with tumour development in either age group.

DISCUSSION

The finding that Walker 256 carcinoma grew to a greater size and had a higher rate of cellular proliferation, in young compared with old hosts is in agreement with the early work of Bischoff and Lang [14]. It has been suggested that two of the factors which may result in this difference are, partial failure of tumour blood supply and nutrition, or an alteration in the non-tumour specific antibody response in the older host [15, 16].

A number of investigators have suggested that neoplasms arise though failure of normal mitotic autoregulation. Wheldon [3] postulated that tumour cells are unable to produce inhibitors, supporting the earlier theory of Osgood [1] that early cell death in tumours results in the lack of differentiation of inhibitor secreting cells. However, subsequently it has been shown that tumour cells do produce

a factor which inhibits mitosis in the equivalent normal tissue type [17] although only low concentrations of this factor are found in malignant compared with normal tissue [18]. This may be a result of inactivation of the inhibitor in the malignant tissue [19]. It has been suggested that failure of malignant cells to respond to this factor is due to a change either in the cell-specific carrier or receptor, resulting in non-recognition of the cell by its tissue specific inhibitor [20].

A previous work has shown that, in the thymus, the mitotic rate increased with increasing cell density to a peak at the mean cell density, but thereafter declined [21]. Similarly, the present findings show that the highest rate of mitosis occurs at the mean cell density, but that at higher densities cellular proliferation is inhibited. This suggests that as cell density increases and extracellular space declines, an inhibitory influence is brought into play.

A model has been proposed to account for this pattern of mitosis assuming that there is a balance between internally produced inhibitors and stimulators of mitosis, related to cell density, which maintains a constant cell mass of proliferative tissue [12]. In particular, high cell densities are thought to be associated with high inhibitor concentrations. This idea fits the present data with regard to the decline in mitosis as the tumour mass ages; the mean mitotic index is reduced at the same time as the mean cell density is increased. The crucial factor in this model is the ratio of extracellular space to cells, in that the larger this ratio, the lower the inhibitor concentration. It may be that, in tumours, the excessive rate of death prevents the major part of the cell population attaining this limiting density, where the extracellular space is reduced sufficiently to increase the concentration of inhibitor to its optimal level. This results in the failure of mitotic autoregulation to stabilize at a fixed cell density. However, this failure of the autoregulatory system does not result in an indefinite growth of the tumour. Rather the tumour stabilizes at a limiting size in the later stages of its growth course, this limitation in tumour size possibly resulting from a failure in the blood supply and nutrients.

With regard to the age of the host it appears that mitosis is more dependent upon cell density in tumours of old hosts. Indeed, generally the mitotic pattern of tumours of old hosts is closer to that observed in normal tissues, implying a better regulation. The differences between the tumours must originate in the age difference between young and old hosts and this will be the basis for future work.

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Weekly Administration of *Cis*-Diamminedichloroplatinum (II) without Hydration or Osmotic Diuresis*

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Abstract—*In order to develop a regimen of* cis-*diamminedichloroplatinum* (II) (*DDP*) *suitable for outpatient use, we treated* 15 *patients with a once weekly dosage schedule of* 40 mg/m^2/week *without prehydration or the induction of an osmotic diuresis. Dose-limiting nephrotoxicity was seen in about* 50% *of the treated patients; myelosuppression was also achieved in a significant number and nausea and vomiting were severe enough to be considered dose limiting in* 4. *Objective antitumor responses were seen in* 6/13 *patients with known or suspected head and neck cancer. We conclude that the present regimen probably produces more renal damage than a comparable dose and schedule given with an osmotic diuresis* (2) *and significantly more subjective distress than intermittent high-dose DDP.*

INTRODUCTION

STUDIES [1] have shown that the nephrotoxicity of *cis*-diamminedichloroplatinum (II) (DDP) can be substantially blunted by a forced diuresis induced by saline and mannitol. This technique has permitted the relatively safe administration of high doses of DDP; unfortunately the procedure used at Memorial Hospital is also a costly one, requiring hospitalization and close supervision of the patient by the professional staff. Recently, Corder *et al.* [2] have developed a modification of this procedure which allows the weekly administration of lower doses of DDP to outpatients. These investigators have also shown a virtually complete absence of clinically significant nephrotoxicity, with myelosuppression the chief dose-limiting side effect.

The present study was undertaken to establish a regimen by which DDP could be used safely in our outpatient department and which might be amenable to combination therapy, once its safety had been established. Since no evidence at present indicated that a forced osmotic diuresis was necessary for protection of the kidneys when relatively modest weekly doses of DDP were used, we decided to begin without such a diuresis.

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MATERIAL AND METHODS

Fifteen patients with far advanced, non-resectable carcinoma were selected for therapy. Criteria for selection included:

1. Histologically confirmed carcinoma
2. Measurable disease
3. Serum creatinine less than 1.2

Prior to therapy, all patients had a complete history and physical examination, complete blood count, twelve-channel screening profile, serum creatinine and chest X-ray. Additional tests were performed only if clinically indicated. Baseline creatinine clearance was measured in most patients.

The treatment program consisted of administration of DDP at a dose of 40 mg/m^2 once a week. On the day of, or the day prior to treatment, the serum creatinine, WBC, hemoglobin and platelet count were determined; treatment was given unless the serum creatinine was greater than 1.4 mg/dl, the WBC <4000, or the platelet count $<150{,}000$. In the event of any of the latter, the treatment was withheld until restitution of normal values. No special effort was made to hydrate the patient prior to drug administration or to encourage diuresis except for an occasional patient whose excessive nausea and vomiting from a previous DDP dose led to apparent dehydration (elevation of BUN with no elevation of creatinine). In that situation patients were given 500 cc D_5 1/2 NS just prior to receiving DDP. In all cases the DDP was given by intravenous infusion over

20–30 min. Response categories are as defined previously [3].

RESULTS

1. *Patient characteristics*

Table 1 shows the distribution of patients with respect to sex, age, primary site and prior therapy. All except for 1 patient had received previous radiotherapy and/or surgical ablative procedure. Only 4 patients had received prior chemotherapy, one with pyrazofurin and the remainder with at least 2 of the following drugs; cyclophosphamide, adriamycin, methotrexate and bleomycin.

Table 1. *Weekly* DDP: *patient characteristics*

Sex:	Male	10	
	Female	5	
Age:	Median	57	
	Mean	59	
	Range	47–76	
Primary Site:	Oro-or hypopharynx	11	
	Unknown primary	2	
	Lung	1	
	Skin appendage	1	
Histology:	Epidermoid	14	
	Adenocarcinoma	1	
Prior therapy:	None		1
	Major surgical ablation		10
	Radiotherapy		11
	Chemotherapy		
	None		11
	CAMB		1
	Polydrug other than CAMB		2
	Pyrazofurin		1

2. *Toxicity*

Of the 15 patients started on therapy with weekly DDP, 4 had therapy stopped after two doses because of rapid tumor progression. These 4 showed minor serum creatinine elevations to less than 1.4 mg/dl as well as minor nausea and vomiting, but no true dose-limiting side effects at the time the drug was stopped. Thus, in the course of therpay, 11 patients achieved some sort of dose-limiting toxicity, which we describe in more detail below.

a. *Nephrotoxicity.* Of the 15 patients who were entered on the study, the incidence of nephrotoxicity is shown in Table 2. In order for a patient to be evaluable for nephrotoxicity, he had to have received the specified number of weekly doses *and* returned one week after the last specified dose for a repeat serum creatinine. Thus, only 13 patients are listed as having received one dose because 2 of the 15 entered did not return on schedule one week after the first dose for a serum creatinine and a repeat dose of the drug. Of the 13 who are evaluable for the effects of a single dose, one developed dose-limiting nephrotoxicity the week immediately following administration of the dose. A second patient had treatment withheld following a cumulative dose of 80 mg/m^2. Two more patients became toxic after three consecutive doses, while another withstood four weekly doses before developing dose-limiting creatinine elevation. Thus, of 11 patients who received weekly DDP to some kind of dose-limiting side effect, 5 had their treatment limited by nephrotoxicity.

When the entire patient group is considered, including those whose weekly treatments were interrupted for dose-limiting side effects other than impaired renal function 10 patients received at least 3 doses of DDP at 40 mg/m^2/dose by day 22 of therapy. Of these, 5 developed at least one episode of dose-limiting nephrotoxicity by day 29. Of 6 patients who received 4 doses of DDP, all within a 29-day span, three patients developed dose-limiting nephrotoxicity. No patient received more than 160 mg/m^2 in consecutive weekly doses because of either nephrotoxicity, myelosuppression, nausea, or disease progression. Two patients did receive a total cumulative dose of 200 mg/m^2 in 6 and 8 weeks respectively and 1 of these developed nephrotoxicity as well. No patient developed a creatinine elevation >2 mg/dl.

b. *Myelosuppression.* Suppression of bone marrow function was also a frequent dose-

Table 2. *Nephrotoxicity from weekly doses*

Number of doses 40 mg/m^2/dose	Number of patients receiving specified number of weekly doses	Number of patients showing nephrotoxicity one week after the last weekly dose	Cumulative number showing nephrotoxicity
1	13	1	1
2	10	1	2
3	7	2	4
4	4	1	5

limiting side effect. As seen in Table 3, 7 of 14 evaluable patients had WBC nadirs below 4000. Five of these 7 patients also had platelet nadirs below 150,000. Only those patients receiving a cumulative dose of DDP of 120 mg/m^2 or greater showed count suppression. Myelosuppression was delayed, with the WBC nadir occurring at a mean of 37 days and platelet nadir at 31 days. In addition, count suppression was generally quite prolonged (mean duration for WBC, 13 days; for platelets, 14 days). No episodes of drug-related sepsis or bleeding occurred in this series. A fall in hemoglobin concentration of >2 g/dl was noted in 7 patients; none manifested clinically apparent bleeding. Drug-induced suppression

Table 3. *Myelosuppression*

WBC nadir	No. of patients	Platelet nadir	No. of patients
>4.0	7	>150	9
3.0–3.9	4	100–149	3
2.0–2.9	2	50–99	2
1.0–1.9	1	<50	0
<1.0	0		

of erythropoiesis appears to be the likely explanation. This manifestation of bone marrow toxicity has been noted in a previous report [3].

c. *Other.* Anorexia, nausea and vomiting were universal reactions to treatment. In most patients the nausea and vomiting lasted only for a few hours on the day of treatment, but in four patients this reaction was severe enough to be considered a true-dose-limiting side effect after 1, 2, 3 and 4 doses of DDP respectively. No patient developed tinnitus, clinically apparent hearing loss, alopecia, or hypersensitivity reactions.

Responses

Objective responses were seen in 6/13 patients with known or presumed head and neck cancer. Of these 6 responses, 1 was a complete response lasting 2 months and 3 were PR lasting 1+, 2 and 3+ months respectively. Two patients showed minor responses of less than 50%. The patient with epidermoid lung carcinoma showed no antitumor response and the patient with a skin appendage primary never received an adequate trial of therapy because of poor compliance. Responding sites included locally and regionally recurrent disease in previously irradiated and/or resected areas as well as more distant metastatic sites.

Table 4. *Weekly DDP: responses to treatment in epidermoid carcinoma of the head and neck*

Patients entered	13
Complete response	1 (2)*
Partial response	3 (1+, 2, 3+)
Minor response	2 (1+, 3)
No change/progression	7

*No. in parenthesis = duration of response in months.

DISCUSSION

We undertook the present study in an effort to establish a convenient and effective schedule of DDP administration, suitable for outpatient use and amenable for combination with other agents. To do this, we explored a dose of DDP which:

(1) is below the dose-limiting nephrotoxic level for the large majority of patients when given every three weeks [4];

(2) has been shown by Corder *et al.* [2] to be non-nephrotoxic when accompanied by a forced diuresis program which, although time-consuming, can be given to outpatients;

(3) adds to approximately the same cumulative dose, if given weekly, as we have explored previously in head and neck cancer patients using an intermittent high-dose schedule with forced diuresis [3]. This dose has also been explored previously by others [5], who found a similar spectrum of toxicities in a small series of 4 patients.

We have found that more than 50% of patients show some evidence of nephrotoxicity when treated according to the present schedule. It may be argued that we should not have regarded creatinine elevations to only 1.4 mg/dl as dose-limiting. Since, however, all but one of the patients had pre-treatment values of $\leqq$1.1 mg/dl, we did not wish to risk further impairment if the serum creatinine had increased by (and, presumably, the glomerular filtration rate had decreased by) nearly 30% of the starting value, until we had verified by subsequent determination that renal function was improving.

We deliberately employed no measures for pre-hydration or diuresis, in order to keep the program as simple as possible. Perhaps even a minimal effort at diuresis or pre-hydration might eliminate some of the nephrotoxicity noted here and yet preserve the convenience of outpatient administration. On the other hand our facilities for outpatient chemotherapy administration are currently strained

to the limit and we felt that, for example, a randomized trial of hydration vs no hydration prior to DDP administration would pose difficult logistical problems. Corder *et al.* [2] have administered DDP in the same dose and schedule as we report here but also employ a vigorous program of forced diuresis with mannitol and intravenous hydration requiring a minimum of 5 hr per patient treatment. These authors report essentially no dose-limiting nephrotoxicity; the mean maximum increase in serum creatinine reported by Corder *et al.* was 0.14 mg/dl (patients received 2–11 treatments, mean 4.6, median 4), while our mean maximal increase in the 11 evaluable patients was 0.53 mg/dl (patients received 2–5 treatments, mean 3.9, median 4). It seems highly likely, therefore, that the Corder regimen conserves renal function more than a similar program withour provisions for hydration and diuresis. Nevertheless, to establish this impression definitely, a prospective randomized comparison of the two methods would be required, with careful matching to insure comparability of the patient groups

In addition to the nephrotoxicity, the nausea and vomiting were a significant feature of this regimen to the patients. When a drug which causes significant emesis is repeated frequently, as here, several hours of emesis has far more significance than it does when the drug is repeated every three to four weeks. At least 4 of our patients regarded the intensity and duration of the vomiting unacceptable and were prepared to discontinue therapy on this account. Our previous experience with high-dose intermittent DDP given once every three to four weeks included *no* withdrawals for this reason.

The antitumor responses to the treatment are noteworthy. Although the numbers are small, the response rate in patients with epidermoid carcinoma of the head and neck (30%) appears comparable to that achieved with the higher dosage schedule of DDP [3] in previously treated patients; the numbers are too small, however, to allow firm conclusions.

In conclusion, our results suggest that weekly DDP does have significant anti-tumor activity at least in epidermoid carcinoma of the head and neck. We feel, however, that the relatively high incidence of nephrotoxicity in this dose and schedule, when the drug is given without a protective diuresis, makes it an impractical agent for combination of methotrexate, whose excretion is almost entirely through the urine and whose toxicity is markedly enhanced in the presence of renal failure. Further efforts to establish a simple, safe and effective outpatients regimen continue.

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The Modification of the Growth Characteristics of the Landschütz Ascites Tumour by Neuraminidase

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Abstract—*The effect of* Vibrio cholerae *neuraminidase (VCN) on the* in vivo *growth characteristics of the Landschütz ascites tumour has been studied. A considerable reduction in tumour cell numbers following the transplantation of VCN-treated cells as compared to untreated cells has been shown which is readily reversible by* in vitro *incubation of the treated cells for a short period prior to transplantation. The possible significance of this is discussed.*

INTRODUCTION

INJECTION of viable tumour cells pretreated *in vitro* with *Vibrio cholerae* neuraminidase (VCN) has been shown to produce immunospecific regression of some chemically induced solid tumours [1–5] and to reduce the transplantability of a number of ascites tumours including the Landschütz tumour [6–10]. We present here a study of the effects of VCN on Landschütz ascites tumour cell proliferation in Schofield albino mice.

MATERIALS AND METHODS

Mice

Eight to twelve week old animals were obtained from a closed colony of random-bred Schofield albino mice maintained at this laboratory.

Tumour

A sub-line of the Landschütz ascites tumour was maintained by weekly intra-peritoneal passage of 0.2 ml of undiluted tumour bearing ascites fluid into healthy female recipients. Tumour cells required for experiment were obtained by collecting ascites fluid into approximately four times its volume of sterile phosphate-buffered saline (P.B.S.) pH 7.2. This prevented clotting without necessitating the use of an anticoagulant. The cells were then sedimented by centrifugation at 150 ***g*** for 5 min and washed three times in fresh P.B.S.

The tumour cell concentration in the washed suspension was determined by visual identification in a haemocytometer. Cell viability was assessed using trypan blue dye exclusion and only suspensions of 90% or greater viability were used. The concentration of viable cells was adjusted to the desired value by the addition of P.B.S.

Neuraminidase treatment

Tumour cells at a concentration of 2×10^7 cells ml^{-1} in Tris buffered saline (T.B.S.) pH 7.2 containing 1 mM $CaCl_2$ were incubated with 5 units of *Vibrio cholerae* neuraminidase (Grade B, 500 Uml^{-1}, Calbiochem) per 10^7 cells at 37°C for 60 min after which the cells were sedimented by centrifugation (150 ***g*** for 5 min) and washed three times in fresh T.B.S. The cells were finally resuspended in phosphate-buffered saline (P.B.S.) pH 7.2 and the cell viability determined by trypan blue dye exclusion. The concentration of viable tumour cells was then adjusted to the desired value.

Tumour cells treated with V.C.N. were suspended in MEM with Earles salts and 25 mM HEPES (Gibco) at a concentration of 10^7 cells ml^{-1} and incubated at 37°C for 3 hr. The cells were then sedimented (150 ***g*** for 5 min) and resuspended in P.B.S., the viability was checked and the concentration of viable cells adjusted to suit.

Assessment of total tumour load

Sheep red blood cells in Alsever's solution (Gibco) were sedimented by centrifugation at 500 ***g*** for 15 min and resuspended in 25 ml P.B.S. plus 0.2 ml of sodium (Cr^{51}) chromate (1 mCi/ml, Radiochemicals). After being gently shaken for 1 hr at room temperature the cells were re-sedimented and washed four times in

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fresh P.B.S. to remove any unbound radioisotope. Tumour bearing animals were injected with 1 ml of this suspension and 10 min later after the red cells were fully dispersed, the animals were sacrificed under ether anaesthesia. The abdominal skin was opened leaving the peritoneum intact and 1 ml of ascites fluid was recovered using a syringe and needle and added to 1 ml of 3.8% trisodium citrate solution. One millilitre of uninjected red cells was treated similarly to act as a standard. Radioactivity counting was performed in a Philips gamma counter.

The tumour cell concentration was determined visually using a haemocytometer and after allowing for various dilution factors, the total tumour load was calculated from the equation

$$\frac{\text{Radioactivity injected (counts/min/ml)}}{\text{Radioactivity recovered (counts/min/ml)}} \times \text{tumour cell count/ml} = \text{total number of tumour cells}$$

This method allowed accurate quantitation of cell numbers down to 5×10^5.

Growth curves

Three groups of 32 male mice were injected intra-peritoneally with 10^7 viable tumour cells per animal. The control group received unmodified cells, the second group received cells freshly treated with V.C.N. and the third group were injected with enzyme-treated cells allowed to stand in tissue culture medium for 3 hr. Growth curves were constructed by determining the total number of tumour cells present in groups of 4 animals at intervals after transplantation.

Statistical analysis

The results obtained with VCN treated cells, either freshly treated or allowed to stand, were compared to the normal values using Student's *t*-test.

RESULTS

The mean (±S.E.) number of tumour cells in each group is shown in Table 1 and Fig. 1.

Growth of untreated cells

Cell proliferation commenced immediately after transplantation and near-exponential di-

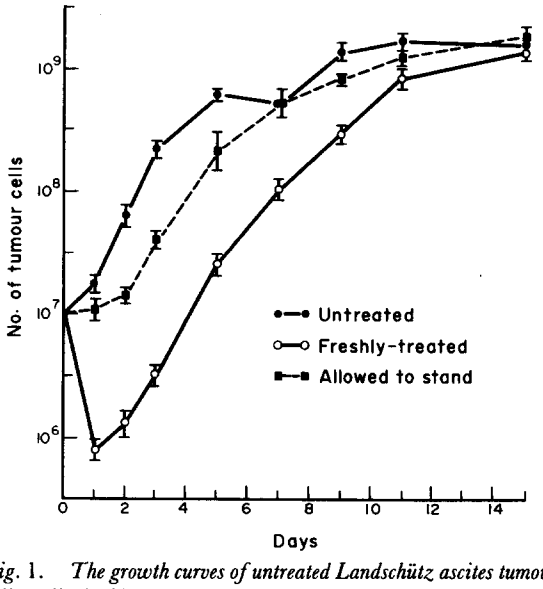

Fig. 1. The growth curves of untreated Landschütz ascites tumour cells, cells freshly treated with VCN and cells allowed to stand in vitro *for a period prior to transplantation.*

Table 1. Tumour cell numbers at intervals following transplantation of unmodified Landschütz ascites cells, cells freshly treated with VCN and cells treated with VCN and allowed to stand before injection (each number = mean of animals ± S.E.)

Time after transplantation (days)	Log_{10} tumour cell numbers (±S.E.)		
	Unmodified cells	Fresh	Allowed to stand
1	7.25±0.06	5.91±0.07	7.04±0.05
2	7.82±0.06	6.14±0.05	7.16±0.08
3	8.36±0.04	6.50±0.07	7.61±0.05
5	8.79±0.02	7.41±0.17	8.35±0.04
7	8.73±0.10	8.04±0.06	8.73±0.06
9	9.13±0.07	8.47±0.03	8.95±0.07
11	9.24±0.05	8.95±0.06	9.12±0.06
15	9.22±0.06	9.17±0.02	9.25±0.05
		$P<0.001$	N.S.

vision (doubling time approximately 20 hr) resulted in the maximum number of tumour cells (2×10^9) being attained eleven days later.

Growth of cells freshly treated with VCN

Although the rate of cell multiplication and maximum cell numbers were comparable with those of untreated cells, a fall in the number of cells to less than 10% of those transplanted resulted in maximum cell number not being attained until fifteen days later. This fall occurred within 24 hr of transplantation and was statistically highly significant ($P<0.001$).

Growth of cells allowed to recover from VCN treatment

No statistically significant difference between these cells and untreated cells was observed (Student's *t*-test).

DISCUSSION

The results presented here show that pretreatment of Landschütz ascites tumour cells by VCN produced a marked retarding effect on the early part of the growth curve when these cells were transplanted into normal mice. This effect was of short duration and the growth curve, after 4 days, paralleled that obtained when normal cells were transplanted indicating that the VCN treated tumour cells retained their ability to proliferate within the host at a rate comparable to untreated cells.

VCN treated tumour cells which were washed and incubated *in vitro* for a relatively short period of time before transplantation showed no difference in growth curve characteristics from untreated cells.

The low recovery of VCN treated tumour cells 24 hr after injection may be due to a number of factors. Tumour cell adherence to the peritoneum may have occurred. It is known that both normal and malignant cells are more adherent after VCN treatment [11]. However, there was no evidence of this in the present experiment and the incidence of solid tumours was similar in mice receiving normal and VCN treated tumour cells. It is possible that VCN treatment impaired viability of tumour cells resulting in transfer of a smaller number of viable cells. Viability was not affected as judged by trypan blue exclusion and it has been shown that much larger concentrations of VCN did not affect cell viability assessed by radioisotope incorporation [12]. Another possibility is that VCN treatment resulted in the exposure of new antigenic sites on the tumour cell membrane with which cytotoxic factors, possibly "natural" antibodies, could react resulting in *in vivo* destruction of the majority of tumour cells [13, 14]. This latter hypothesis is supported by the observation that *in vitro* culture of VCN treated tumour cells for a short period of time reversed the observed effect possibly by allowing regeneration of removed membrane components with recovery of normal antigenicity. Finally it is known that VCN remains bound to treated cells despite repeated washings and this cell bound VCN may have as yet undefined *in vivo* effects which may explain the observed difference in growth curve characteristics [15].

The ability of VCN treated Landschütz ascites cells to proliferate *in vivo* in an immunologically competent host is at variance with a previous report [9]. Differences in enzyme concentration and incubation conditions are unlikely to be responsible for this discrepancy since it has been observed that near maximal removal of sialic acid residues is obtained under conditions employed in the present study [16].

VCN treatment enhances the immunogenicity of many tumours [1–10]. Proposed mechanisms for the enhancement of immunogenicity include alteration of membrane deformability facilitating phagocytosis [11] and exposure to neoantigens and/or the modification of existing antigens resulting in the induction of a strong and cross reactive immune response against unaltered antigens [7]. The rapid reduction in tumour cell numbers which follows transfer of VCN treated tumour cells raises the possibility that one mechanism of action of VCN may be to predispose these cells to attack by preexisting defences resulting in a dramatic reduction in tumour load below numbers required for a successful take.

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Influence of Vasoactive Drugs and Ischaemia on Intra-Tumour Blood Flow Distribution

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Abstract—*The intra-tumour blood flow distribution in two transplantable rat tumours was studied by isotope technique. Intravenous infusion of noradrenaline reduced significantly the blood flow distribution in both intramuscularly transplanted tumours and in normal muscle. This effect was counteracted by pre-treatment of animals with an alpha-receptor blocking agent, phenoxybenzamine. Tumour and muscle blood flows were moderately increased after ischaemia.*

INTRODUCTION

NO ADRENERGIC innervation of tumour vessels was found in two intramuscularly transplanted rat tumours, in spite of topographically good vascular connections with normal vessels of the transplantation area in at least one of the tumours, a sarcoma [1]. This observation, which is in accordance with a previous study [2] does, however, not exclude alpha-adrenergic receptors in tumour vessels and the possibility to influence the tumour blood flow by vasoactive drugs acting on these receptors.

In previous studies the tumour blood flow was found to be increased after local X-ray irradiation of the same rat tumours. Furthermore, a post-ischaemic increase of the total relative tumour blood flow was found by plethysmography [3]. Other recent experimental studies have also suggested that the tumour blood flow might be influenced by vasoactive drugs [4, 5].

The aim of the present study was to investigate the influence on the intra-tumour blood flow distribution in the two earlier studied rat tumours by ischaemia, by noradrenaline and by an alpha-receptor blocking agent, phenoxybenzamine.

MATERIALS AND METHODS

Animals

Inbred rats from a Lister strain of animals and with a mean body weight of 150 g were studied.

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Tumours

A 20-methylcholanthrene induced sarcoma (MCH-H) in its 125th to 129th transfer generations and a hepatoma (Hep-H) in its 212th to 213th transfer generations were studied. These tumours have been described in detail previously [3]. Tumours were transplanted intramuscularly into one hindleg of the animals by a trochar technique and the experiments were performed two weeks after tumour transplantation with a mean tumour diameter of 15 mm.

Noradrenaline

Inj-Noradrenaline 1 mg/ml (Apoteksbolaget, Sweden). One millilitre of a saline solution with 0.01 mg noradrenaline/ml was slowly injected into the lower caval vein during 5 min. This injection was immediately followed by isotope injection.

Phenoxybenzamine

Dibenyline® (Smith, Kline & French Lab. Ltd, England). Five tenth mg/100 g body weight in 0.5 ml saline was injected intravenously into a tail vein 60 min before the experiment [6].

Isotope

^{86}Rubidium (^{86}Rb), dissolved in distilled water to an isotonic solution, was injected intravenously in a dose of 500 μCi and a volume of 0.1 ml. It was obtained from Kabi Diagnostica, Studsvik, Sweden.

Ischaemia

The aorta and lower caval vein were clamped through a lower transverse abdominal incision under ether anaesthesia for 10 min. Blood flow distribution was studied within 3 min after removal of clamping.

Recording of the intra-tumour and intra-muscle blood flow distribution

The intra-tissue blood flow distribution was studied by an isotope technique according to Sapirstein and recently described in detail [3]. Five hundred μCi ^{86}Rb in a volume of 0.1 ml was injected intravenously into the lower caval vein through a transverse abdominal incision under ether anaesthesia. A larger isotope dose than in the previous study was used due to the radiation properties of ^{86}Rb [3].

In previous studies [3] a stable isotope concentration plateau was reached in tumour about 20 sec after isotope injection and this stable plateau was maintained for several min. During this period the uptake and release of isotope balance each other despite recirculation and the fractional uptake of isotope within the tissue will reflect the intra-tissue distribution of blood flow as a fraction of cardiac output according to Sapirstein (see [3]).

The aorta and lower caval vein were clamped 60 sec after isotope injection. Tumours and normal muscle tissue from the contralateral hindleg were rapidly dissected and frozen in expanding carbon dioxide. Multiple 10–50 mg frozen specimens from each tumour and from normal muscle in each animal were taken for radioactivity recording. The isotope dose injected into animals was dissolved in 150 ml saline and three 1 ml specimens were taken as standards.

Tumour and muscle samples and standards were examined in a well-type scintillation counter with a pulse analyzer. Due corrections were made for background. The recorded activities in most tissue samples were 10^3–10^4 counts/min.

All results were calculated and are presented as % of injected dose/g tissue. This figure indicates the percentage of cardiac output/g tissue and should make possible a comparison between tumour and muscle blood flow if Sapirstein's conditions are fulfilled.

Blood pressure registration

The influence of ischaemia, noradrenaline and phenoxybenzamine on the mean arterial blood pressure was studied in three separate animals. Blood pressure was recorded continuously under ether anaesthesia by a polyethylene catheter (PE 50) in the left carotid artery, connected with a Grass polygraph recorder with a Statham pressure transducer.

RESULTS

Intravenous infusion of noradrenaline induced an insignificant increase of the mean arterial blood pressure, while phenoxybenzamine decreased the arterial blood pressure slightly (5–10 mm Hg). A normal blood pressure (125 ± 5 mm Hg) in phenoxybenzamine injected animals was restored by noradrenaline infusion.

The flow data presented for each experimental group are collected data from five animals in which about 20 tumour specimens and 15 muscle specimens were taken from each animal. A wide distribution of ^{86}Rb was found in tumours and in normal muscle. The isotope uptake was strongly reduced in both tumours and in normal muscle after infusion of noradrenaline. This reduction was counteracted by pre-treatment of animals with phenoxybenzamine. A moderate increase of isotope uptake was found in muscle and in tumours after ischaemia (Fig. 1a and b).

DISCUSSION

In the present study the intra-tumour blood flow distribution was recorded by an isotope technique. Two transplantable rat tumours with topographical differences in vascular supply were studied. Infusion of noradrenaline reduced the intra-tumour blood flow distribution in both tumours significantly as in normal muscle. This reduction was counteracted by pre-treatment of animals with an alpha-receptor blocking agent, phenoxybenzamine. Tumour and muscle blood flows were moderately increased by ischaemia.

The observations made in this study might suggest that tumour blood vessels could be influenced by vasoactive drugs acting on alpha-receptors. However, the similar response in tumour and normal muscle to infusion of noradrenaline might also indicate that changes of blood flow distribution in tumours are secondary to induced changes in normal tissue blood flow distribution in the transplantation area.

The possibility to influence tumour blood flow is of large clinical interest. Vasoactive drugs have been used in pharmacoangiography to enhance the contrast filling of tumour vessels. Thus, e.g. vasoconstriction by angiotensin or by epinephrine of normal vessels was suggested to

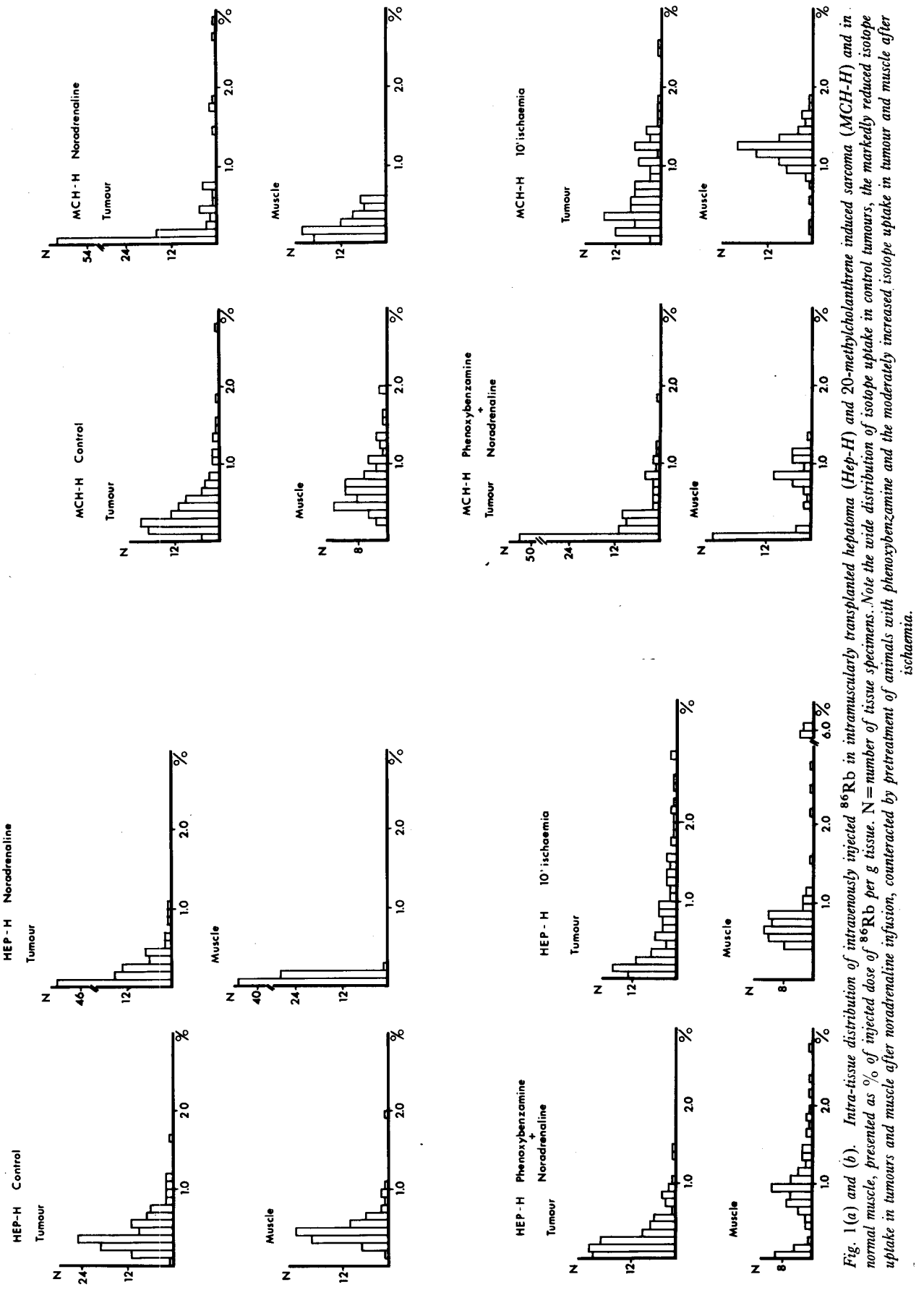

Fig. 1(*a*) *and* (*b*). *Intra-tissue distribution of intravenously injected* ^{86}Rb *in intramuscularly transplanted hepatoma* (*Hep-H*) *and 20-methylcholanthrene induced sarcoma* (*MCH-H*) *and in normal muscle, presented as % of injected dose of* ^{86}Rb *per g tissue.* N = *number of tissue specimens. Note the wide distribution of isotope uptake in control tumours, the markedly reduced isotope uptake in tumours and muscle after noradrenaline infusion, counteracted by pretreatment of animals with phenoxybenzamine and the moderately increased isotope uptake in tumour and muscle after ischaemia.*

stand out in contrast to non-reactive tumour vessels and to enhance the contrast filling of tumour vessels [7, 8].

The observations made in the present experimental studies indicate that tumour blood flow might be markedly influenced by vasoactive drugs. However, this effect on tumour blood flow might be a direct effect on alpha-receptors in tumour vessels, but might also be secondary to an influence on normal blood vessels in the transplantation area. This will be further studied. Topographical differences in vascular morphology between the tumours did not influence changes in tumour blood flow. The increased intra-tumour isotope distribution after ischaemia is in accordance with earlier observed increased total relative tumour blood flow by plethysmography after ischaemia [3].

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Consequences of Inhibition of Purine Biosynthesis *de novo* by 6-Methylmercaptopurine Ribonucleoside in Cultured Lymphoma L5178Y Cells*

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Abstract—*6-Methylmercaptopurine ribonucleoside is an inhibitor of amidophosphoribosyltransferase and produces growth inhibition and loss of viability in cultured murine lymphoma L5178Y cells. Treatment with low concentrations of this drug leads to lowered concentrations of purine ribonucleotide concentrations, whereas those of pyrimidine ribonucleotides are elevated. At higher drug concentrations the concentration of the latter are also decreased. Concentrations of both purine and pyrimidine deoxyribonucleoside triphosphates are lowered by drug treatment. Treated cells lose protein, RNA and DNA into the medium.*

INTRODUCTION

6-METHYLMERCAPTOPURINE ribonucleoside (MeMPR||), in the form of its 5′-monophosphate, is an inhibitor of the first enzyme of the pathway of purine biosynthesis *de novo*, amidophosphoribosyltransferase (EC 2.4.2.14); there is good evidence that this is the primary, if not sole, site of enzymatic action of MeMPR in animal tumor cells (review: [1]. Previous studies [2–5] have shown that treatment with MeMPR results in alterations in the intracellular concentrations of purine and pyrimidine ribonucleotide concentrations; in particular, the concentrations of ATP and GTP decrease appreciably whereas those of UTP and CTP increase. In the present work, we have made further attempts to learn how inhibition of purine biosynthesis *de novo* by MeMPR leads to inhibition of cell growth and to loss of cell viability. In particular, we have studied further the effects of MeMPR treatment on purine and pyrimidine ribonucleotide concentrations, and have also measured its effects on purine and pyrimidine deoxyribonucleotide concentrations. In addition, the loss of proteins and nucleic acids from MeMPR treated cells has been detected.

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||Abbreviation used: MeMPR, 6-methylmercaptopurine ribonucleoside.

MATERIALS AND METHODS

MeMPR and purine and pyrimidine bases, nucleosides and nucleotides were supplied by Sigma Chemical Co., St. Louis, MO. Sucrose (density gradient grade) was supplied by Schwarz/Mann, Orangeburg, NY. [Methyl-^{3}H]thymidine (3 Ci/mmole), [8-^{3}H]hypoxanthine (10 Ci/nmole), and L-[2-^{14}C] leucine (46 mCi/mmole) were obtained from Schwarz/Mann, Orangeburg, NY and [1,2-^{14}C]glycine (101. mCi/mmole), from New England Nuclear Corp., Boston, MA.

The murine lymphoma L5178Y cells used in this study were grown in stationary suspension culture in Fisher's medium supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, NY). Stock cultures were maintained in logarithmic phase by dilution at least every 48 hr to a density of *ca.* 0.2×10^5 cells per ml. In the experiments reported here, the average generation time was 11 hr. Cell densities were measured using a Model Z_f Coulter Counter.

The incorporation of radioactive precursors into perchloric acid-insoluble material was measured as described previously [6]. After 3 washes with cold perchloric acid, the acid-insoluble material was dissolved in 0.1 N NaOH and radioactivity was measured in a liquid scintillation counter using Bray's scintillation fluid [7].

Purine biosynthesis *de novo* was measured by determining the extent of incorporation of [1,2-^{14}C]glycine into total cellular adenine and guanine, as described previously [6].

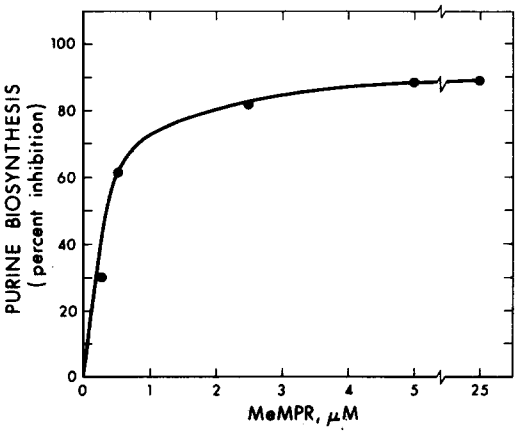

Fig. 1. Effect of MeMPR on Purine biosynthesis de novo. *Lymphoma* L5178Y *cells,* 0.94×10^5 *cells per* ml, *were incubated with various concentrations of MeMPR and* 100 μM [^{14}C]*glycine for* 30 min, *after which the incorporation of radioactivity into total cellular adenine and guanine was measured. These data are representative of results obtained in four experiments.*

Concentrations of acid-soluble purine and pyrimidine ribonucleotides were determined spectrophotometrically after their separation by high-pressure liquid chromatography using a Reeve Angel Partisil-10SAX column (25 cm × 4.6 mm; Mandel Scientific, Montreal, Que.) and a linear gradient of potassium phosphate and potassium chloride, pH 4.5, as described in detail elsewhere [8].

Concentrations of deoxyribonucleoside triphosphates were measured enzymatically, using a modification of the procedure originally developed by Solter and Handschumacher [9] as modified by Lowe and Grindley [10]. The assay was linear within the range of 2 to 50 pmole of each deoxyribonucleioside triphosphate.

Changes in DNA structure were detected by determining the molecular weight of DNA single strands by alkaline sucrose density gradient centrifugation by the method of Walters and Hildebrand [11].

To prepare cells for electron microscopy, they were collected by brief centrifugation, the medium was removed, 1 ml of the cells were suspended in 5% phosphate buffered glutaraldehyde fixative and then centrifuged. The pellet was dehydrated in a series of ethanols, embedded in Epon, sectioned,

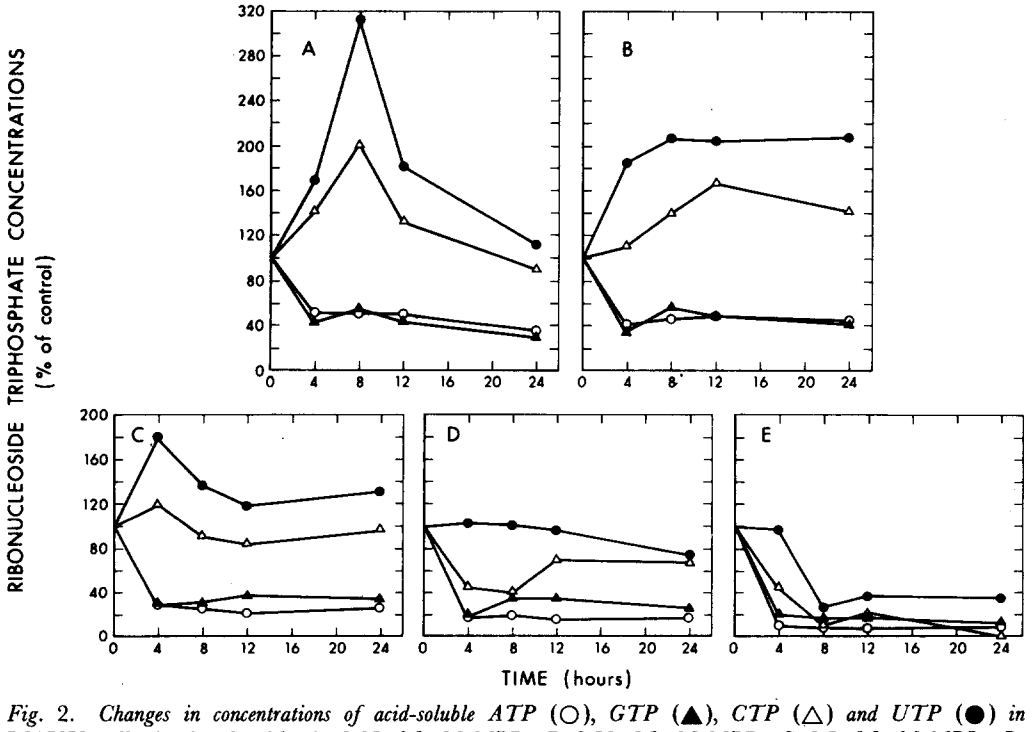

Fig. 2. Changes in concentrations of acid-soluble ATP (○), *GTP* (▲), *CTP* (△) *and UTP* (●) *in* L5178Y *cells incubated with A,* 0.25 μM *MeMPR, B* 0.50 μM *MeMPR, C* 2.5 μM *MeMPR, D,* 5.0 μM *MeMPR and E,* 25 μM *MeMPR. Values for control (untreated cells) were ATP,* 1932 nmole/10^9 *cells: GTP,* 406 nmole/10^9 *cells: UTP,* 772 nmole/10^9 *cells and CTP,* 334 nmole/10^9 *cells. These data are representative of those obtained in two experiments.*

double stained with uranyl acetate and lead citrate and examined using a JEM-7A electron microscope.

RESULTS

Initial studies were carried out to establish the relationship between MeMPR concentration and its primary biochemical effect, inhibition of purine biosynthesis *de novo* and between MeMPR concentration and growth inhibition. Figure 1 shows that inhibition of purine biosynthesis increased from *ca.* 30 to 90% as MeMPR concentrations were increased from 0.25 to 5.0 μM; further increases produced no further inhibition. Other studies showed that inhibition of purine biosynthesis by MeMPR declined slowly with time. For example, 0.5 μM MeMPR produced 62% inhibition immediately after addition of drug to the culture, but only 46% inhibition 8 hr later. Because of this variation with time it is difficult to establish the exact relationship between inhibition of purine biosynthesis *de novo* and inhibition of growth. However, an approximation is obtained by measuring cell growth 24 hr after addition of the same MeMPR concentrations as those used in Fig. 1. Control cells grew from a starting density of 5×10^4 cells/ml to a density of 17×10^4/ml after 24 hr. Cultures treated with 0.25, 0.5 and 2.5 μM MeMPR grew from the same starting density to 13.5, 9.8 and 5.2×10^4/ml, respectively. Cell numbers decreased to 4.0 and 3.0 $\times 10^4$/ml, respectively, in cultures treated 24 hr with 5.0 and 2.5 μM MeMPR. (Detailed growth curves were obtained, but are not reported here).

In order to study the immediate biochemical consequences of MeMPR treatment, we next studied the changes in intracellular concentrations of purine and pyrimidine ribonucleotides that resulted from treatment with this drug. Figure 2 reports concentrations of ribonucleoside triphosphates* both as a function of time of incubation with drug and as a function of MeMPR concentration. Concentrations of ATP and of GTP decreased within 4 hr at all concentrations of MeMPR used. In general ATP and GTP concentrations decreased to approximately the same relative extent in most cases, although there were some small differences. The magnitude of the initial decrease in purine ribonucleoside triphosphate concentrations increased with increasing MeMPR concentrations, from *ca* 40% of control at 0.25 μM MeMPR to *ca.* 10% of control at 25 μM MeMPR.

Concentrations of UTP and CTP increased following treatment with lower concentrations (0.25, 0.5, 2.5 μM) of MeMPR, although this increase lagged behind the decrease in ATP and GTP concentrations; the increase was greatest in cells treated with the lowest concentrations of MeMPR. MeMPR concentrations of 5 and 25 μM lead to decreases in pyrimidine ribonucleoside concentrations, with CTP concentrations declining more than those of UPT.

In order to obtain further information regarding the effects of MeMPR on nucleotide metabolism, intracellular concentrations of purine and pyrimidine deoxyribonucleoside triphosphates were measured. Figure 3 shows that concentrations of both dATP and dGTP decreased substantially within 2 hr of initiation of treatment with 25 μM MeMPR, and that

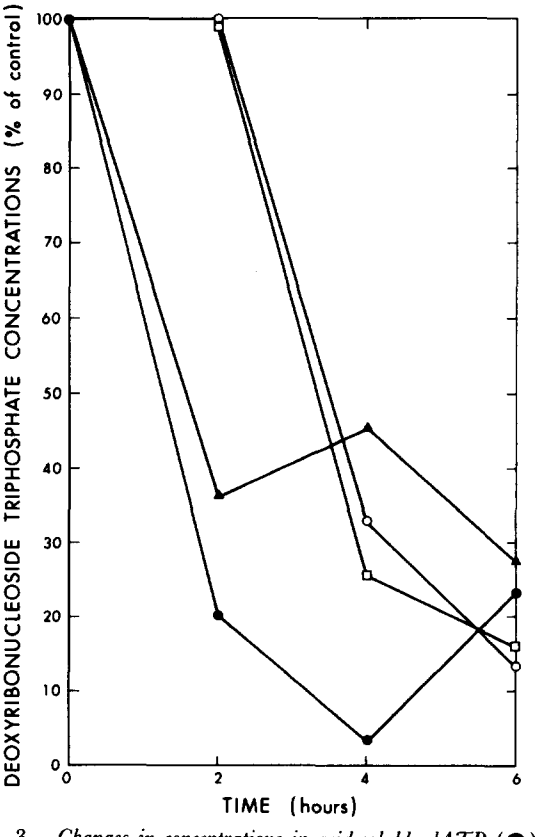

Fig. 3. Changes in concentrations in acid-soluble dATP (●), dGTP (▲), dCTP (□) and TTP (○) in L5178Y *cells treated with* 25 μM *MeMPR. Values for control (untreated cells) were dATP,* 15 nmole/10^9 *cells; dGTP,* 8 nmole/10^9 *cells; dCTP,* 98 nmole/10^9 *cells; TTP,* 28 nmole/10^9 *cells. These data are representative of those obtained in two experiments.*

*Concentrations of the purine and pyrimidine ribonucleoside mono- and diphosphates were less than 10% those of the corresponding triphosphates at all times. For convenience and brevity, and because the triphosphates quantitatively are the most important nucleotides, only the concentrations of the latter are reported here.

by 4 hr, concentrations of dCTP and TTP had also dropped appreciably.

The studies described so far provided a reasonable basis for understanding the growth inhibitory effects of MeMPR. However, these data by themselves did not appear to be sufficient to explain the loss of cell viability (3, 4 and unpublished observations) that is produced by treatment with this drug. The possibility that macromolecules were breaking down in cells treated with MeMPR was therefore considered. In these experiments, lymphoma L5178Y cells were first grown with a radioactive precursor, transferred to fresh medium without precursor for sufficient time to reduce acid-soluble radioactivity to low levels and then treated with MeMPR. At various times the cells were collected by centrifugation, and the amount of radioactive material that had passed from the cells into medium was measured. A portion of medium was treated with cold 0.4 M perchloric acid and the percentage of the total radioactivity in the medium that was acid-insoluble was calculated. [^{14}C]Leucine was used as a precursor of proteins, [^{3}H]hypoxanthine as a precursor of combined RNA plus DNA and [^{3}H]thymidine as a precursor of DNA.

Figure 4 shows that treatment with relatively high concentrations of MeMPR led to greatly accelerated loss of cellular protein, even as early as 4 hr after initiation of treatment. By 8 hr, *ca.* 3-times as much radioactivity was lost from treated cells as from untreated cells. More than 40% of the cellular radioactivity lost into the medium was acid-precipitable even at 2 hr and in the MeMPR treated cells this increased to *ca.* 80% by 8 hr.

Figure 5 shows the effects of a wide range of MeMPR concentrations in the total loss of radioactivity in cellular nucleic acids (RNA plus DNA) over the course of 24 hr treatment. In control cells, less than 10% of total cellular nucleic acid radioactivity was lost in 24 hr. Treatment with MeMPR, however, led to an accelerated loss that was time and concentration dependent. After treatment with relatively high concentrations of MeMPR for 24 hr, between 58 and 67% of the initial cellular nucleic acid radioactivity was lost into the medium. Further studies showed that a substantial proportion of the radioactivity lost of cells treated with MeMPR was derived from DNA.

Because substantial loss of RNA, DNA and protein was lost from MeMPR-treated cells, they were examined by electron microscopy in attempts to detect morphological consequences of such losses. However, no consistent differences between control and drug treated cultures were noted.

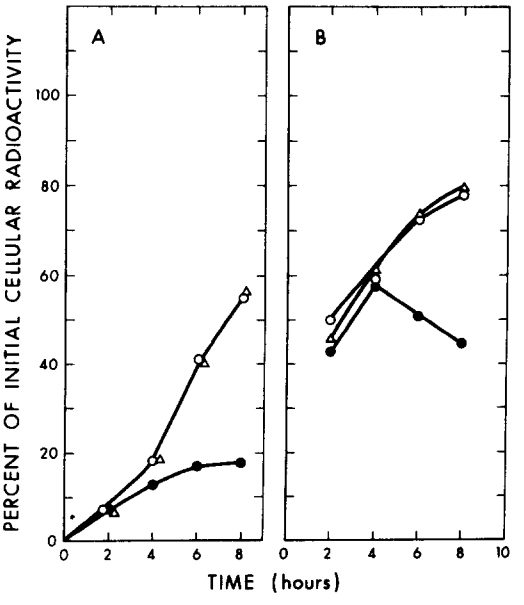

Fig. 4. Loss of radioactivity from cellular protein in lymphoma L5178Y *cells treated with MeMPR. Lymphoma* L5178Y *cells were grown for* 18 hr *with* 0.1 μCi/ml [^{14}C]*leucine. Cells were then transferred to fresh medium without precursor and grown for an additional* 4 hr. *The cell density was then adjusted to* ca. 10^5 *cells per* ml *and growth was continued for* 8 hr *with no addition* (●), 25 μM *MeMPR* (○), *or* 37.5 μM *MeMPR* (△). *At various times, samples were taken and radioactivity in the medium was determined. A, total radioactivity in the medium as a percentage of the total radioactivity originally present in the cells. B, acid-insoluble radioactivity in the medium as a percentage of the total radioactivity in the medium. These data are representative of those obtained in three experiments.*

DISCUSSION

In this study we have attempted to gain further information regarding the biochemical and biological consequences of inhibition of amidophosphoribosyltransferase in cultured lymphoma L5178Y cells. Although the line of L5178Y cells used in these studies appeared to be less sensitive to growth inhibition by MeMPR than that previously used by Warnick and Paterson [4], these authors added fresh drug daily, whereas we did not; the two types of experiments therefore cannot be directly compared.

As a consequence of inhibition of purine biosynthesis *de novo* by MeMPR, intracellular concentrations of purine ribonucleotides decreased. This result has of course been reported by other investigators [3, 4, 5], but this study has measured ribonucleotide concentrations at a wider range of MeMPR concentrations and times of drug treatment than

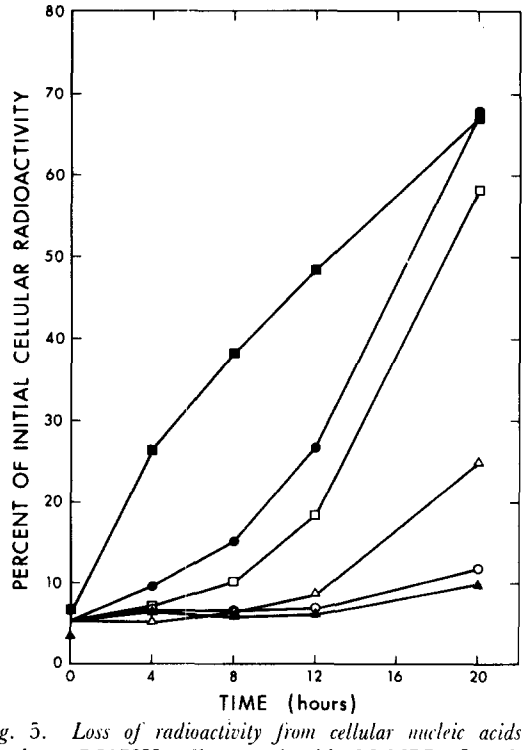

Fig. 5. Loss of radioactivity from cellular nucleic acids in lymphoma L5178Y *cells treated with MeMPR. Lymphoma* L5178Y *cells were grown with* 0.1 μCi/ml [^{3}H]*hypoxanthine as described in Chart 7 and finally grown with* 0 *MeMPR* (▲), 0.375 μM *MeMPR* (○), 0.75 μM *MeMPR* (△), 3.75 μM *MeMPR* (□). 7.5 μM *MeMPR* (●), *or* 37.5 μM *MeMPR* (■). *Results are calculated as total radioactivity in the medium as a percentage of the total radioactivity originally present in the cells. These data are representative of those obtained in two experiments.*

reported previously. The relative concentrations of purine ribonucleoside triphosphates were reduced to as low as 10% of control values as a result of treatment with 25 μM MeMPR. MeMPR treatment has previously been shown to produce elevated concentrations of pyrimidine ribonucleotide concentrations [3–5], particularly at low drug concentrations. However, it has not previously been observed to cause these concentrations to decrease at high levels of drug.

Decreased concentrations of purine ribonucleotides appeared to lead directly to decreased synthesis of purine deoxyribonucleotides, whose concentrations decreased rapidly following treatment with MeMPR. Concentrations of pyrimidine deoxyribonucleoside triphosphates decreased to almost the same extent; effects of MeMPR on deoxyribonucleoside triphosphate concentrations have not previously been reported.

The consequences of inhibition of amidophosphoribosyltransferase so far described would appear to be sufficient to explain the growth inhibitory effects of MeMPR. However, growth inhibition appears to be followed by loss of cell viability and this is more difficult to explain in biochemical terms. We have shown here that treatment of lymphoma L5178Y cells led to appreciable losses of protein, RNA and DNA from the cells, and that much of the material lost still was acid precipitable; i.e. was not broken down to free amino acids and nucleotides, nucleosides and bases. Although it may be supposed that these changes in MeMPR treated cultures are related to the observed decrease in apparent cell survival, it is not yet established whether they are causes or consequences of cell death. This point and the relationship of intracellular nucleotide concentrations to the processes of protein and nucleic acid breakdown, obviously require further study. It is of considerable interest, however, that reductions in GTP and dGTP concentrations in cells treated with mycophenolic acid, do not lead to such drastic catabolism of macromolecules [12]. Such catabolic processes, therefore, are not automatic concomitants of cytotoxicity.

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Adaptation of Murine MOPC-315 Myeloma Cells to Growth *In Vitro* and Further Characterization of their C-Type Viruses

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Abstract—*MOPC-315 murine myeloma cells were successfully adapted to growth in culture and the established cell line propagated in suspension and retained the capacity to induce plasma cell tumors in mice. The cultured myeloma cells constantly release RNA viral particles which by nucleic acids hybridization are indistinguishable from the C-type particles secreted into the ascitic fluid of mice bearing MOPC-315 myeloma tumors. Availability of an* in vitro *source of MOPC-315 murine myeloma viral particles facilitated additional biochemical characterization of these viruses. The myeloma particles were found to be infectious and capable of inducing syncytia in XC cells. The particles released in culture, unlike those secreted* in vivo, *showed high instability during the purification steps, but this was partially overcome by the addition of dithiothreitol.*

INTRODUCTION

IN A PREVIOUS paper [1] we reported that MOPC-315 murine myeloma cells secreted *in vivo* C-type particles which, unlike other myeloma viruses [2, 3], possess biochemical properties similar to those of known RNA tumor viruses. Although a high yield of viral particles can be obtained from the ascitic fluids of tumor-bearing mice, this *in vivo* source is inconvenient and rather costly. The tumor cells, for instance, have to be transplanted weekly and this necessitates a regular supply of animals. During such *in vivo* sequential transplantation, the myeloma cells are prone to contamination by other viruses of the host or, accidentally, by viruses from other animals kept in the same animal house. Viral preparations of *in vivo* sources also contain a high proportion of "old" virions with degraded molecular components as well as large amounts of contaminating cellular substances, rendering them unsuitable for determination of viral molecular constituents [4]. In addition to the mentioned drawbacks, there is also the difficulty in studying various aspects of the reproductive cycle of myeloma C-type particles.

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In view of all the above, we deemed it profitable to render MOPC-315 tumor cells amenable to growth in tissue culture. As will be shown in the present paper, we found that the viral particles released by the tumor cells during their replication *in vitro* were identical to those released by them *in vivo*. The availability of an *in vitro* source of MOPC-315 viral particles enabled further biochemical and biological characterization of these particles.

MATERIALS AND METHODS

Preparation of MOPC-315 cell line

A subcutaneous tumor induced in BALB/c mouse by MOPC-315 cells [5], was excised under sterile conditions and washed several times in phosphate-buffered saline (PBS), pH 7.2. The tumor specimen was minced with sharp scissors, then suspended in Eagle's modified medium and forced through a fine stainless-steel mesh inserted in a 30 ml syringe in order to obtain single cells. Following removal of tissue fragments by filtration through gauze, the cells were washed, resuspended in Eagle's modified medium supplemented with 10% calf serum (CS) and seeded in 75 cm^2 plastic (Nunc) flasks at a concentration of 0.5×10^6/ml. The cultures were incubated at 37°C in humidified air with 5% CO_2. Cells were subcultured at 3-day in-

tervals in a vol of 15 ml per flask at a concentration of 2×10^5 cells/ml.

DNA polymerase assay

Reaction mixtures (0.1 ml) for homopolymer-templated reactions contained the following: Tris–HCl (pH 8.2), 50 mM; potassium chloride, 48 mM; magnesium chloride, 8 mM; dithiothreitol (DTT), 0.4 mM; dATP, TTP(P.L. Biochemicals), 0.2 and 0.1 mM respectively; ^{3}H-TTP (40 to 50 Ci/mmole, New England Nuclear Corp.) to yield 300 counts/min/pmole and synthetic oligo$(dT)_{12-18}$ poly (rA) (P. L. Biochemicals), 0.4 μg. For assaying the endogenous enzymatic activity the reaction mixtures (125 μl) contained 100 μg/ml actinomycin D; 50 mM Tris-HCl (pH 8.2); 10 mM NaCl; 4 mM $MnCl_2$; 1.2 mM each of dGTP, dCTP, dATP and 600 μCi/ml of ^{3}H-TTP. Virus particles in 0.01 M Tris-HCl (pH 8.2) were disrupted in the presence of 0.05% Nonidet P-40 (NP-40) at 0°C for 10 min and then the appropriate reaction mixture was added. Reactions were incubated at 37°C and terminated as described in the text. Acid-precipitable radioactivity was tested after precipitation with cold trichloroacetic acid (TCA) at a final concentration of 10% and collection on nitrocellulose filters. Following exhaustive washings with cold 5% TCA, filters were dried and the radioactivity counted in 0.4% 2,5-bis-2(5-tert-butylbenzoaxozolyl) thiophene (BBOT) in toluene.

Isopycnic purification of particles

Medium was harvested 48 hr after seeding MOPC-315 cells at a concentration of 2×10^5 cells/ml. Medium was clarified from cells by centrifugation at 2000 ***g*** for 10 min and further centrifuged at 10,000 ***g*** for 10 min. The supernatant fluids were then centrifuged through 20% (v/v) glycerol in TNE (0.01 M Tris–HCl (pH 7.4), 0.1 M NaCl, 0.001 M EDTA) for 1 hr at 95,000 ***g*** in a Beckman SW-27 rotor. The pellet was resuspended in TNE, layered on a gradient of 20–55% (w/w) sucrose in TNE or on a glycerol gradient (1.12–1.24 g/ml) and centrifuged at 95,000 ***g*** for 16 hr at 4°C. The gradient was fractionated and densities were determined by refractive index measurement. Fractions were tested for DNA polymerase activity or for ^{3}H-radioactivity and those corresponding to the viral peak were pooled, diluted with TNE and sedimented at 95,000 ***g*** for 1 hr.

Preparation of cores

MOPC-315 cells were grown in the presence of ^{3}H-uridine (5 μCi/ml, 20 Ci/mmole) for 48 hr. Purified viral particles, concentrated from the 1.15 to 1.18 g/ml density region of a sucrose gradient, were resuspended in TNE and treated with NP-40 at a final concentration of 0.5%, in the presence of 0.1 M DTT, for 5 min at 0°C. The resulting solution was layered on a 20–65 (w/w) sucrose-TNE gradient and centrifuged at 100,000 ***g*** for 18 hr at 4°C. Fractions were collected from below and assayed for acid-precipitable radioactivity.

Isolation of viral RNA

Purified viral particles concentrated from isopycnic gradients were resuspended in TNE and treated with 1% SDS for 5 min at room temperature. The suspension was then deproteinized twice with an equal volume of phenol-cresol-8-hydroxyquinoline and the RNA was purified by centrifugation on a 10 to 30% (v/v) glycerol gradient, at 234,000 ***g*** for 90 min. The RNA-containing fractions were pooled and the nucleic acid was precipitated by the addition of two volumes of cold alcohol in the presence of 0.3 M LiCl.

Synthesis and purification of [^{3}H] *cDNA*

[^{3}H] thymidine-labelled cDNA was synthesized in an endogenous reverse transcriptase reaction with detergent-disrupted viruses in the presence of actinomycin D. The reaction was carried out at 37°C until the kinetics of ^{3}H-TMP incorporation reached a plateau level. The [^{3}H]DNA product was deproteinized and further purified by chromatography on G-50 Sephadex column equilibrated with 1 mM EDTA containing 0.2 M sodium bicarbonate. The peak radioactive fractions in the excluded volume were pooled, lyophilized and the RNA template was alkali-digested.

Hybridization conditions and analysis

Annealing reactions between [^{3}H]cDNA (1000 counts/min) and various amounts of viral 70 S RNA were performed in 10 μl reaction volume containing 0.06 M NaCl, 0.04 M Tris–HCl pH 7.2, 0.002 M EDTA and 0.1% SDS. The reaction mixtures were sealed in siliconized capillaries, kept at 100°C for 2 min, chilled on ice and then incubated at 68°C for varying periods of time to reach the desired Crt values. Hybrids were analyzed by re-

sistance to S1 nuclease [6] in the following manner. After incubation, samples were withdrawn, adjusted to 0.03 M sodium acetate (pH 4.5), 0.3 M NaCl, and 3 mM $ZnSO_4$ containing 10 μg/ml of denatured *M. luteus* DNA. This was equally divided into two tubes, to only one of which was added a titrated quantity of S1 nuclease (Miles Laboratories). Following incubation at 37°C for 1 hr, the nucleic acids were precipitated, collected on filters and the radioactivity measured in a liquid scintillation spectrometer. Data were plotted as percentage hybridization vs Crt to determine the $Crt_{1/2}$ values and the final extent of hybridization. Background counts corresponding to 5% or less of the input counts/min were subtracted from each time point.

Infectivity assay

BC wild mouse embryo clone SC-1 cells [7], in Eagle's modified medium containing 10% FCS, were seeded into plastic tissue-culture cluster plate (Costar) at a concentration of 5×10^5 cells/well (16 mm dia). After 24-hr incubation, cells were treated with DEAE-dextran (25 μg/ml, Pharmacia) for 1 hr, washed with culture medium and then infected with 0.05 ml MOPC-315 virus stock obtained from culture media filtered through 0.45 μm Millipore filters. Following 2 hr adsorption at 37°C, the viral inoculum was removed, the cells were washed and 1 ml of fresh medium was added. Cultures were incubated for 3 days at 37°C, then 2×10^5 DEAE-dextran-treated XC cells were added to each well. After 3 more days of incubation, the cells were fixed with methanol, stained with Giemsa and scanned for syncytia with an inverted light microscope.

RESULTS

Adaptation of MOPC-315 cells to growth in culture

As mentioned earlier (Materials and Methods) the MOPC-315 cell line was established from a subcutaneous tumor of BALB/c mouse. The tumor cells easily adapted to the *in vitro* environment and demonstrated a high growth rate in Eagle's modified medium supplemented with 10% calf serum. The cultured MOPC-315 cells are growing in suspension and at the time of writing are at the 210th generation having completed two years of cultivation *in vitro*. The growth curve of the myeloma cell line reveals a generation time of 19 hr and a saturation density of 10^6 cells/ml which is reached after 72 hr of growth. The tumorogenic potential of various generations of the MOPC-315 cell line was tested in BALB/c mice and it was found that upon intraperitoneal or subcutaneous injection of 10^6 cells, all animals developed plasma cell neoplasms within 10 days.

Optimal conditions for virus production

The viral yields from MOPC-315 cell line were estimated by the level of templated DNA polymerase activity in extracellular particles. The optimal cell concentration for maximal virus production was determined from the quantity of particles in tissue culture fluids of MOPC-315 cells seeded at various concentrations and grown for 72 hr. For this purpose, the particles were concentrated from 10 ml amounts of cell free tissue culture fluids, by sedimentation through 20% glycerol and assayed for DNA polymerase activity. Data obtained (Table 1) showed that the highest yield of particles was produced by MOPC-315 cells seeded at a concentration of 2×10^5 cells/ml.

Table 1. *DNA polymerase activity in culture fluids of MOPC-315 cells seeded at various concentrations*

Initial cell density/ml	pmole TMP incorporated*
1×10^5	67.4
2×10^5	158.9
3×10^5	121.3
4×10^5	23.4

*Assay performed as described in "Materials and Methods" on 100,000 *g* pellet from 10 ml clarified cell culture medium. The pmole values represent activity after 30 min reaction in the presence of oligo dT. poly rA template.

The next aim was to determine the optimal period of incubation necessary for maximal yield of particles. Towards this end MOPC-315 cells were seeded in 75 cm^2 plastic tissue culture flasks at a concentration of 2×10^5 cells/ml, then incubated at 37°C and the yield of particles possessing DNA polymerase activity measured after various periods of incubation. It was found (Table 2) that 48 hr of incubation was optimal for virus harvest.

Stability of MOPC-315 viruses in sucrose and glycerol gradients

The buoyant density of MOPC-315 viral particles was determined in sucrose–TNE and

Table 2. Kinetics of the release of particles with DNA polymerase activity from MOPC-315 cells

Virus harvest (hr)	pmole TMP incorporated*
24	117.0
48	214.2
72	22.4
96	7.0

*MOPC-315 cells were seeded at a concentration of 2×10^5 cells/ml and incubated at 37°C. At the indicated hours, culture fluid (10 ml) was harvested and processed as in Table 1.

glycerol–TNE equilibrium gradients. High-speed pellets obtained from cell-free culture fluids were subjected to isopycnic density centrifugation, the gradients then fractionated and aliquots tested for templated DNA polymerase activity. In sucrose and glycerol gradients, the particles banded at densities of 1.16 and 1.19 g/ml respectively (Fig. 1). It was further revealed that unlike the particles released into the ascitic fluids of mice bearing myeloma tumors [1], the MOPC-315 particles were extremely unstable in these gradients. However, when 3 mM DTT was incorporated into the gradient solutions, the viral particles acquired considerable stability, particularly in glycerol gradients (Fig. 1b).

Cores of MOPC-315 viral particles

To determine the density characteristics of the viral core component of myeloma particles, a "density-shift" experiment was performed. ^{3}H-uridine labelled particles were concentrated from the 1.15 to 1.18 g/ml density region of a sucrose gradient, treated with nonionic detergent and centrifuged to equilibrium in a 20–65% sucrose–TNE gradient, containing 3 mM DTT. The outcome of this experiment (Fig. 2) demonstrated that the detergent treatment released from MOPC-315 particles the internal nucleoid having a buoyant density of 1.26 g/ml. Recovery of the viral cores was law and most of the labelled material was found in the upper part of the density gradient. Evidently the core components of myeloma viruses are highly sensitive when freed from their outer membranes.

Sedimentation coefficient of virion RNA

To determine the sedimentation coefficients of the RNA species characteristic for MOPC-315 viral particles, the nucleic acid was ex-

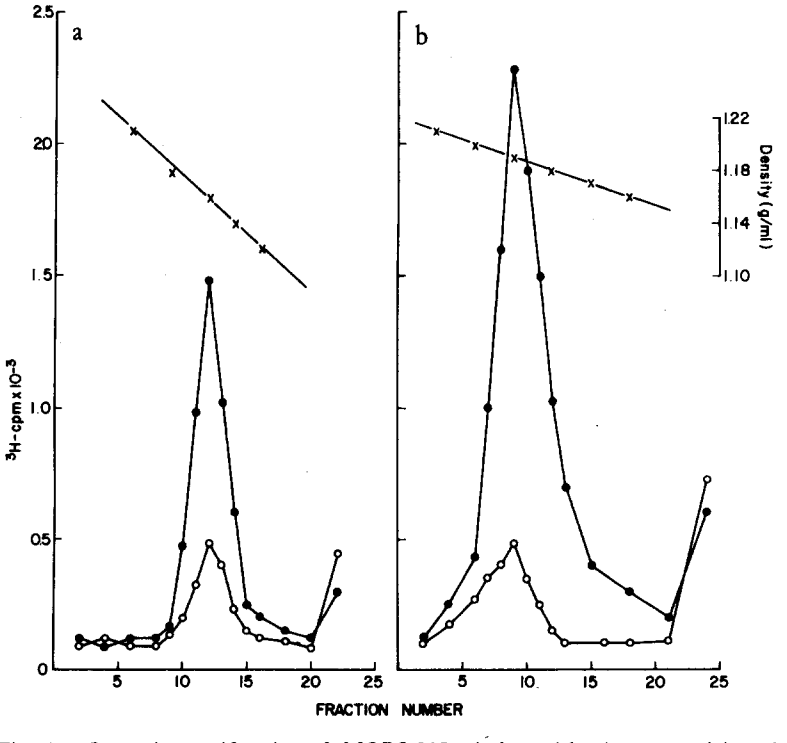

Fig. 1. Isopycnic centrifugation of MOPC-315 viral particles in sucrose (a) and glycerol (b) gradients with [●---●] or without [○---○] DTT. High speed pellets of clarified culture fluids were centrifuged to equilibrium and 20 µl aliquots of gradient fractions were tested for templated DNA polymerase activity. Reaction mixtures were incubated for 30 min at 37°C and assayed for acid-precipitable radioactivity as described in "Materials and Methods".

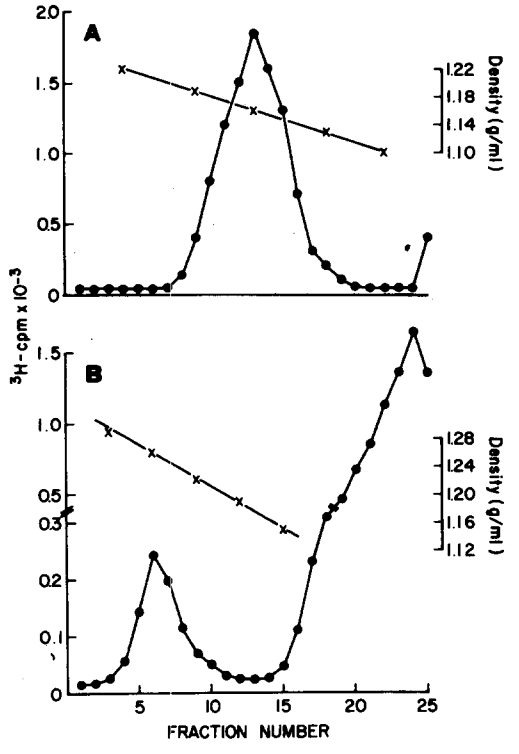

Fig. 2. Conversion of MOPC-315 viral particles to viral cores. ^{3}H-uridine labelled particles were centrifuged to equilibrium in 20–55% sucrose gradient. 20 µl aliquots of the gradient fractions were assayed for acid-precipitable radioactivity (A). Particles concentrated from the 1.15 to 1.18 g/ml density region were treated with NP-40 and subjected to isopycnic sucrose density centrifugation as described in the text. 30 µl aliquots of the gradient fractions were then tested for acid-precipitable radioactivity (B).

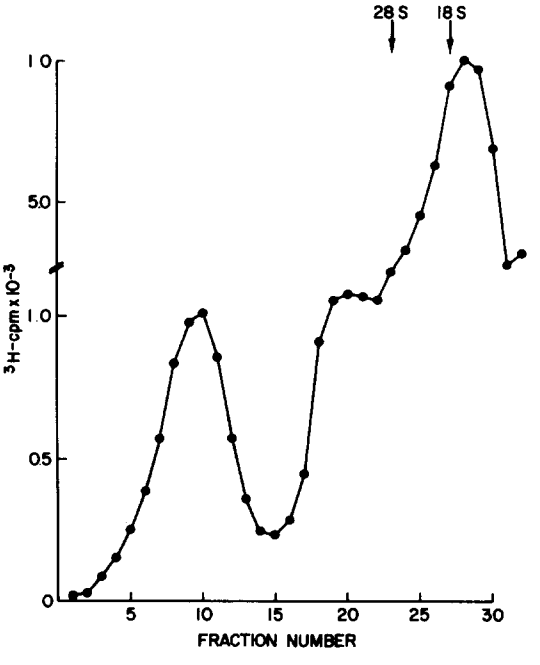

Fig. 3. Rate zonal centrifugation profile of MOPC-315 viral RNA. The nucleic acid was extracted from purified ^{3}H-uridine labelled particles and centrifuged in 10–30% glycerol gradient at 234,000 g for 90 min. Fractions were collected and assayed for acid-precipitable radioactivity. The external markers were 18 and 28 S ribosomal RNA.

tracted from purified ^{3}H-uridine labelled particles and analyzed in a 10–30% glycerol gradient. Two major species of RNA molecules were detected (Fig. 3): a heavy 70 S RNA and a lighter 15 S RNA. Occasionally 35 S RNA molecules, possibly degraded fragments of the high molecular weight RNA, were also detected.

Homology of MOPC-315 particles produced in vitro *to the one obtained from ascitic fluids*

Hybridization kinetic analyses were performed to establish the relatedness of the viral particles secreted by cultured MOPC-315 cells to the particles released *in vivo* by the parental cells. [^{3}H]cDNA prepared in an endogenous reaction of the viral particles isolated from tissue culture fluids of MOPC-315 cells was annealed at identical Crt values to 70 S RNAs extracted from viral particles of both sources (Fig. 4). The fact that both RNA species followed the same hybridization kinetic curve, the nearly identical and low $Crt_{1/2}$ values ($\sim 2.5 \times 10^{-1}$ mol/sec/liter), as well as the final

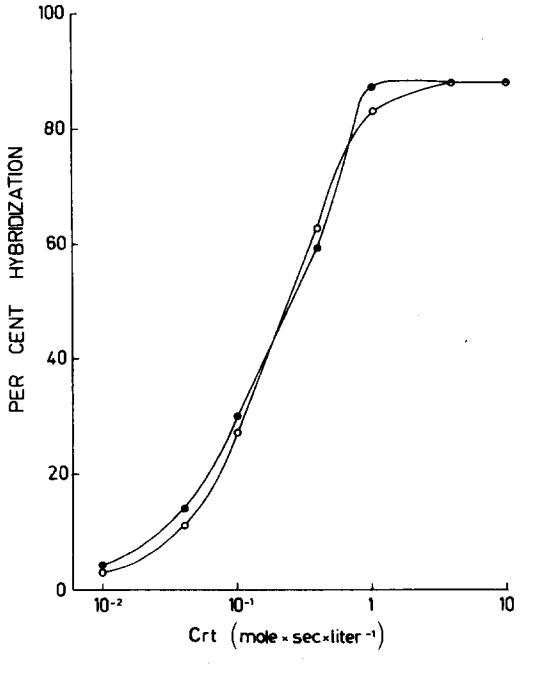

Fig. 4. Hybridization kinetic analysis of RNAs from viral particles produced by MOPC-315 cells grown in vitro (●) *or* in vivo (○). [^{3}H]*cDNA was synthesized in an endogenous reaction of viral particles obtained from cultured MOPC-315 cells. At the indicated Crt values the hybridization reaction was terminated and assayed for the acquisition of S1 nuclease resistance as described in "Materials and Methods".*

extent of hybridization (88%), all suggest that the nucleotide sequences of the examined viruses are indistinguishable.

Infectivity of MOPC-315 viral particles

The infectious potential of the viral particles produced by MOPC-315 cell line was assessed on SC-1 cells known for their high sensitivity to infection by ecotropic viruses of mouse origin [8]. The productive infection was determined by syncytia-inducing effect on XC cells. Syncytia with as many as 10–60 nuclei/cell were observed (Fig. 5). They contained vacuoles and usually were surrounded by a clear area of faintly stained cytoplasm. No syncytia were observed in control cultures.

When [^{3}H]cDNA of viruses produced by cultured MOPC-315 cells was annealed to 70 S RNA obtained from viral particles released by MOPC-315 cells or SC-1 infected cells, very similar $Crt_{1/2}$ values of about 2.2 $\times 10^{-1}$ mole/s/liter were detected. This indicates that the viral particles secreted by the infected SC-1 cells are generally related to the murine myeloma MOPC-315 viruses.

DISCUSSION

C-type particles have been isolated from several cultured murine myelomas [2, 3, 9] and although in all these instances, the viruses were derived from plasma cell tumors of BALB/c mice, they were found to differ in respect to buoyant density, RNA species and presence or absence of RNA-instructed DNA polymerase. In fact, in some of their biochemical features they differ also from the known murine oncornaviruses.

We have previously shown [1] that unlike other murine myeloma type C particles, the viral particles released *in vivo* by MOPC-315 tumor cells possess the ordinary characteristics of murine C-type viruses, i.e. a buoyant density of 1.16 g/ml in sucrose gradients, 60 to 70 S RNA and an RNA-instructed DNA polymerase that transcribes efficiently the viral RNA. The adaptation of MOPC-315 tumor cells to growth *in vitro* has enabled further bio-chemical and biological characterization of the viral particles produced by these myeloma cells. The *in vitro* secreted viruses, like those produced by FLOPC-1 cells [2], are unstable in sucrose and glycerol-TNE gradients. Such instability was not observed in the viral particles secreted *in vivo* by MOPC-315 cells, although the viral genome in both instances was indistinguishable as shown by our hybridization studies. Possibly the higher protein content or other substances in the ascitic fluids of tumor-bearing mice, exert a stabilizing effect on the myeloma C-type particles. A similar difference has been reported also in the case of Rous sarcoma virus where the particles contained in tumor extracts were found to be much more resistant than ones in the tissue culture supernates [10]. Volkman and Krueger [2] were able to partially stabilize the viral particles released from cultured FLOPC-1 cells by using gradients of glycerol in EBSS. Such gradients, however, did not exert any stabilizing effect on MOPC-315 particles. On the other hand, the addition of a reducing agent (DTT) conferred a substantial protective effect on MOPC-315 viral particles, probably by preventing the oxidation effect known to be largely responsible for inactivation of viral particles during their purification [11].

None of the C-type particles isolated thus far from murine myeloma cells has demonstrated any oncogenic potential. Possibly this inability to induce malignancy stems from the biochemical defects that had been detected in them. MOPC-315 viral particles, in contrast, are fully infectious as well as biochemically nondefective. Within the murine myeloma C-type viruses they are therefore a suitable candidate for testing whether this group of viruses is also potentially malignant. This possibility is now being investigated.

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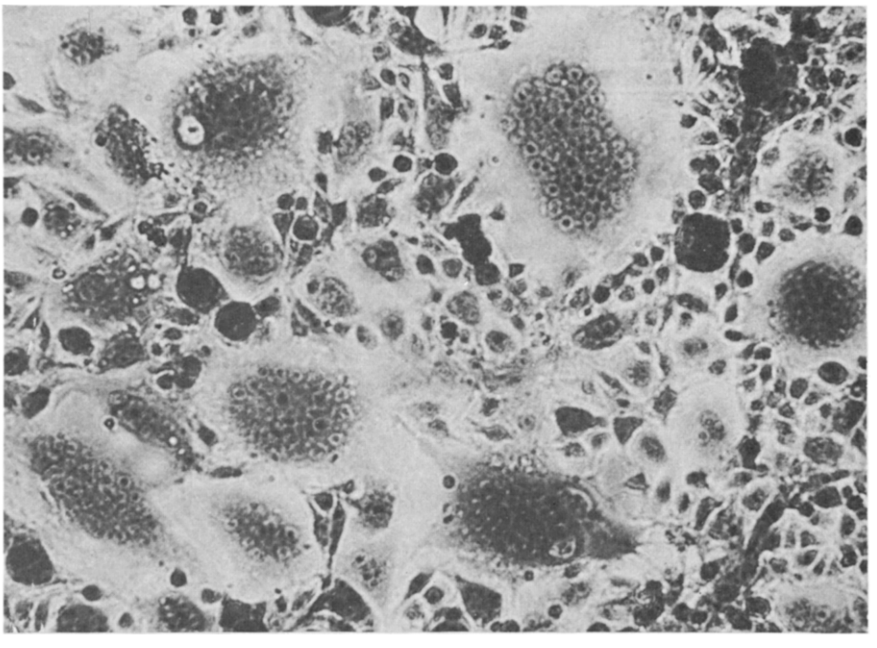

Fig. 5. *Syncytia induced by co-cultivation of SC-1 cells infected with MOPC-315 viral particles and XC cells. The XC cells were added on day 3 post infection and stained with Giemsa on day 6.* ×240.

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Functional Evaluation of Pulmonary Bilateral Irradiation Effects

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Abstract—*Early systematic bilateral pulmonary irradiation is to be considered with interest in patients with tumours which have a high metastatic risk for the lungs. Theoretical considerations show that this irradiation can sterilize from 15 to 30% of infra-radiological pulmonary metastases.*

This whole-lung irradiation is feasible provided that the dose does not exceed 2000 rad in 10 sessions of 200 rad over 2 weeks or 1500 rad in 4 sessions of 375 rad over 7 days (both regimens being equivalent to NSD = 870 rets).

The functional pulmonary deficit is negligible for such a dose but it will be advisable to exclude contraindications such as a chronic respiratory insufficiency or the association of some chemotherapies known as noxious to the lungs.

INTRODUCTION

BILATERAL pulmonary irradiation can be used to prevent the evolution of microscopic metastases potentially present in lungs after curative treatment of tumours giving a high percentage of pulmonary metastases [1–7].

From this standpoint the Radio-Chemotherapy Group of EORTC has studied through a controlled clinical trial the "prophylactic" value of a whole-lung irradiation of 2000 rad in patients treated for osteosarcoma. Breur *et al.* will report the results of this trial [8].

The aim of the present study is to analyze the effects of such a dose on the respiratory functions of 21 cases treated in the Centre François Baclesse [9]. These cases have been submitted to functional pulmonary exploration tests before and after irradiation, at regular intervals, over a period ranging from three months minimum to two years for a few patients.

MATERIALS AND METHODS

Selected patients

Patients entering this study were affected with various types of cancer having a high tendency for metastasizing to the lungs (Table 1).

Primary tumours received preliminary radical treatment (radiotherapy and/or surgery).

Table 1

Localization	Number of cases	Combination chemotherapy
Soft tissue Sarcomas	12	Actinomycin (500 μg X 5) 2 cases
Renal tumour	8	No
All others	3	Cytoxan 1 case

Only three patients had received moderate chemotherapy.

Radiological examination of the lungs, as well as functional exploration and blood-gas were normal before irradiation.

Throughout the follow-up and, in most cases, afterwards, no metastasis occurred radiologically.

Irradiation method

Irradiation was given with Cobalt 60, at a SAD of 80 cm.

The two lungs were irradiated entirely from the top to costodiaphragmatic sinuses without mediastinal protection. Two anterior and posterior opposed fields were used, the two fields being irradiated at each session.

Four doses of 375 rad were delivered over seven days, giving a total dose of 1500 rad calculated at mid-plane in the middle of each lung (Fig. 1).

Accepted 2 December 1977.

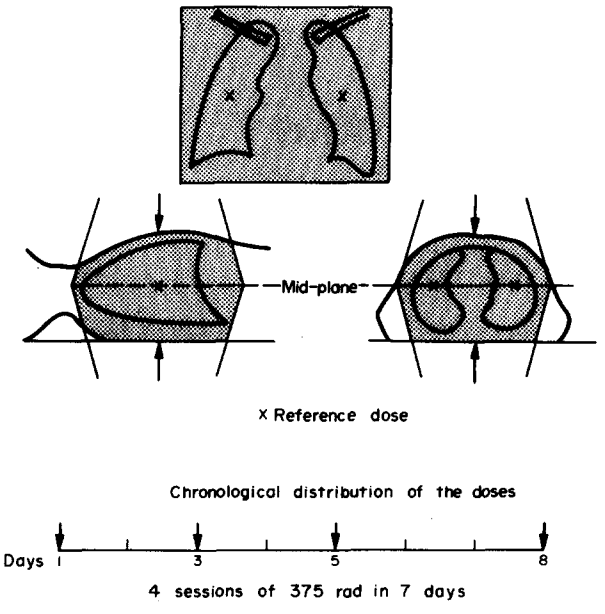

Fig. 1. Target volume and chronological distribution of the doses.

An air correction has been made for the lungs. According to Ellis formula, this treatment corresponds to 870 rets, that is, a dose equivalent to 2000 rad in 10 sessions over 14 days [10].

Functional tests consisted of: classical spirography (vital capacity, residual volume) and a study of carbon oxide transfer by steady state technique with calculation of total and partial ductances recommended by Lacoste [11]. This functional study was completed by an analysis of arterial blood-gas.

Theoretical values used were those of CECA for the spirometry, those of Dechoux and Pivoteau [12] for the carbon oxide transfer.

Chronology of examinations

The protocol included a complete functional study with measurement of blood-gas and radiological control the day before the irradiation. Successive tests took place on the 21st, 90th, 180th, 240th and 300th days. For a few patients, the tests could be continued after two years (three years for two patients).

The number of patients studied appears in Table 2.

RESULTS

General clinical and radiological tolerance was revealed to be good, confirming our previous results [1, 9] and those of many authors.

A slight dyspnea on exertion could however be noted in three patients on the 21st, 90th and 180th days, but never beyond the 6th month.

One patient who did not present clinical signs rasied a radiological alarm when pulmonary micronodules appeared on the 6th month after irradiation. This patient tested 2 yr later, presents neither any late noticeable functional effects, nor any radiological sequellae.

Functional tolerance has been revealed to be satisfactory as is shown by the evolution of the parameters in Table 2 and in the Figs. 2–4.

The pulmonary volumes are not modified and the observed variations (not significant) never exceeded −15%.

Table 2

	Days after radiotherapy	21	90	182	240	300	364	728	990	1200
	n	19	13	14	7	6	2	2	2	2
DuaCo	M	−2.8	−7.8	−4.3	−8.5	−10.8	−14.4	−12.7	−0.6	−10
(% of theoretical	s	4.7	10.7	10.2	8.7	11.4	9	26	5	18
values)	*P*	0.02	0.02	0.14	0.04	0.07	0.27	0.61	0.91	0.58
	n	21	14	14	9	8	2	2	2	2
PaO2	M	−2.7	−5.4	−5.7	−6.7	−4.6	−9	−4.5	−13	1.5
(mm Hg)	s	9	11.4	7	12.6	8.5	7	16.3	18.4	6.4
	P	0.19	0.10	0.01	0.15	0.17	0.32	0.76	0.5	0.8
	n	20	19	12	11	7	—	2	2	—
Vital capacity	M	−3.9	−6.8	−14.3	−13.4	−13.2	—	—	—	—
(% of theoretical	s	10.9	19	18.6	17.5	19.6	—	—	—	—
values)	*P*	0.12	0.14	0.02	0.03	0.13	—	—	—	—

n = number of study patients.
M = average.
s = standard deviation.
P = significance probability of observed deviation.

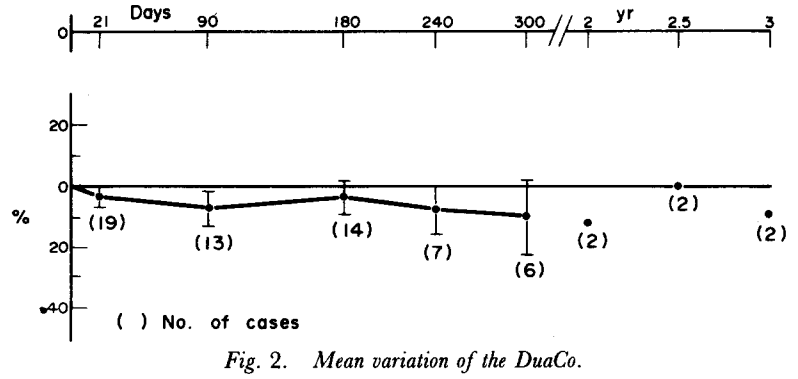

Fig. 2. Mean variation of the DuaCo.

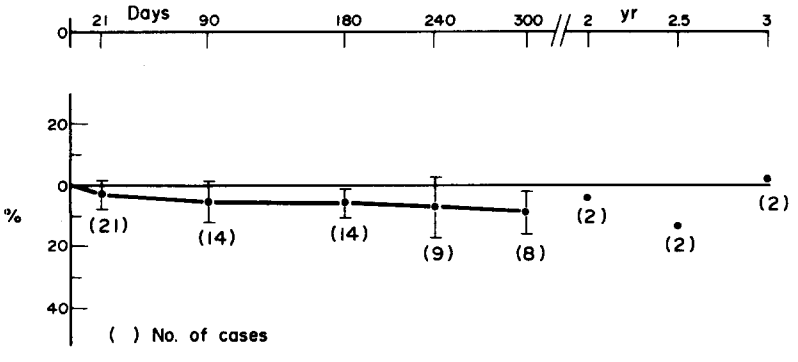

Fig. 3. Mean variation of the PaO2.

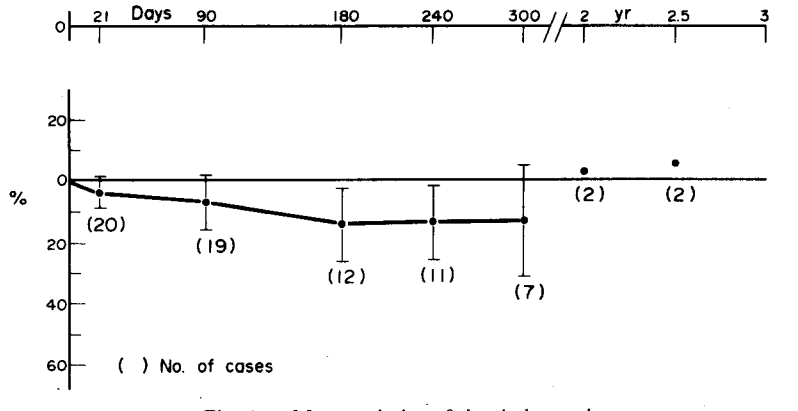

Fig. 4. Mean variation of the vital capacity.

The same is true for the PaO2, which decreased to 6 mmHg on the 180th day ($P = 0.01$).

The functional parameter whose modification is the most significant is without any doubt the alveoloarterial ductance (DuaCo), which explores the flow from the alveolar gas to the blood of the pulmonary capillary. This ductance decreased significantly ($P = 0.02$) from the 21st day, reaching $-8\% \pm 8\%$ on the 90th day ($P = 0.02$) and stabilized thereafter around -10% (standard deviation = 5 – 11%) (Table 2).

These functional modifications, although significant, can be considered to be negligible and do not seem to increase beyond one year's follow-up (Figs. 1–3). However, our material is too limited to allow a definitive conclusion beyond this period.

DISCUSSION

Functional tests can be said to have disclosed an effect of radiotherapy at the selected dose on the lung tissue. This dose can be considered as tolerable as long as pulmonary function is normal before radiotherapy.

The functional picture is very similar to that described by Emirgil [13] and Boushy [14] in man and those of Sweany [15] and

Teates [16] in animals: these authors observed a very pronounced restriction of pulmonary volumes and an important perturbation of gaseous exchanges for irradiation doses around 4000–5000 rad. Sweany investigated the effects of a whole-lung irradiation in animals with a dose of 4000 rad over 8 weeks (260 kV), and found a 54% drop in carbon oxide transfer capacity within 137 days. The observed deficiencies as well as the given doses are without any relationship with those that were stated in the present study.

Other authors performed more or less extensive irradiations with doses ranging from 1380 to 1838 rets. They observed carbon oxide transfer modifications ranging from 17 to 50% [17, 18].

Most of the studies in man concern patients with pulmonary metastases, making functional exploration interpretation difficult.

From the results presented here, it seems inadvisable to deliver doses above NSD 870, which probably can be regarded as the maxima.

Association with chemotherapy requires the radiation-dose to be decreased if the drugs which are used are known to have an action on pulmonary parenchyma [19].

In patients with respiratory deficiency, pulmonary irradiation is contra-indicated if ventilatory deficit exceeds 20% or if there are gaseous exchange disorders. However, in practice, it is necessary to weight the risk of lung metastases and that of inducing respiratory deficiency.

From a carcinological point of view, concerning the effects of such an irradiation, calculations show that 10 sessions of 200 rad over 14 days can destroy up to 30% of the infraradiological pulmonary metastases [2, 4]. It is even possible that the selected fractionation (4 sessions of 375 rad), also equivalent to 870 rets, has an increased effect on tumour cells [20]. The results obtained in the centre François Baclesse are encouraging, especially in soft tissue sarcoma [21].

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Hemodialysis and Enzymatic Cleavage of Methotrexate in Man*

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Abstract—*Renal insufficiency prolongs the serum half-life of methotrexate (MTX) and results in toxicity unless MTX can be removed, detoxified or its biochemical effects reversed. We studied the ability of hemodialysis and* in vivo *enzymatic cleavage with carboxypeptidase G_1 ($CPDG_1$) to reduce the serum MTX concentration in a patient with leukemia and renal failure. On three trials, a* 5 hr *hemodialysis failed to increase the serum MTX clearance or remove significant amounts of drug. On two trials, intravenous injection of $CPDG_1$ caused an immediate* 1.0–1.3 log *unit fall in the MTX concentration measured by a kinetic enzyme assay, but only a minor change in MTX measured by radioimmunoassay. This difference is accounted for by cross-reaction of the product of the enzymatic cleavage of MTX with the antibody used in the radioimmunoassay. This study demonstrates the inability to remove MTX from serum by hemodialysis in patients with high serum levels of MTX and poor renal function and defines some of the difficulties associated with enzymatic detoxification.*

INTRODUCTION

THE COMBINATION of high dose methotrexate (MTX) and leucovorin rescue is an important chemotherapeutic regimen with significant activity in osteogenic sarcoma [1], lymphoma [2] and carcinoma of the head and neck [3]. Significant response rates have also been reported for other human neoplasms [4–6]. The major toxicities of the MTX/leucovorin program are myelosuppression, mucositis and acute renal failure. Since MTX is excreted primarily by the kidneys [7], in the presence of preexisting renal disease or acute renal damage due to the drug itself, the serum half-life is prolonged and this may result in inadequate rescue by standard doses of leucovorin [8]. In such situations, life-threatening marrow and gastrointestinal toxicity is imminent unless serum MTX concentrations can be rapidly lowered, or the biochemical effect of MTX can be reversed.

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We report here the results of trials, in a patient with severely imparied MTX excretion, of hemodialysis and enzymatic detoxification of MTX using carboxypeptidase G_1 ($CPDG_1$), a bacterial enzyme that rapidly cleaves the terminal glutamate from MTX.

MATERIALS AND METHODS

Patients

The patient was a 50-yr-old male with chronic myelogenous leukaemia in blast crisis resistant to all standard chemotherapeutic drugs. He had developed acute renal failure three months earlier in association with an episode of septic shock for which he was treated with cephalothin and gentamycin. At the time of these studies, he had a creatinine clearance of less than 1% of normal and required tri-weekly hemodialysis with a Cordis/Dow 1.3 m^2 hollow fiber kidney model 4 to maintain a serum creatinine of 5 to 12 mg/dl. Dialysis was performed at the Peter Bent Brigham Hospital Dialysis Center using regional heparinization.

Because of a rising blast cell count the patient was given three courses of MTX therapy at biweekly intervals at doses of 130, 130 and 400 mg/m^2 administered as a rapid intravenous pulse. On all three courses, thymidine rescue consisting of a continuous infusion of 8 g/m^2/day was started 24 hr

after the injection of MTX and continued until MTX levels measured by radioimmunoassay were less than 5×10^{-8} M. Dialysis studies were performed on the first course of therapy, and during the first 24 hr after MTX injection on the second course. Trials of $CPDG_1$ were carried out on the second and third courses. Written informed consent for these studies was obtained.

MTX and thymidine levels were measured by radioimmunoassay [9, 10]. In the studies involving $CPDG_1$, MTX levels were determined by both radioimmunoassay and kinetic enzyme assay [11]. EDTA 1 mM was added to freshly drawn blood samples to a final concentration of 0.1 mM. We found that this concentration of EDTA completely inhibited $CPDG_1$ and prevented cleavage of MTX during sample transfer and preparation (Uren, J., unpublished observations). The kinetic enzyme assay was performed using *L. Casei* dihydrofolate reductase.* Conditions of the assay were Tris Cl, 50 mM; NADPH, 80 μM; bovine serum 10%; dihydrofolate reductase 2.5×10^{-3} units/ml; dihydrofolate 50 μM; pH 7.4; 37°C. The enzyme was preincubated for 5 min at 37°C with the inhibitor and reaction mixture minus the dihydrofolate, and the reaction was initiated by the addition of dihydrofolate. Percent inhibition was calculated from the ΔOD_{340}/min determined over the initial 10 min of the reaction. In reaction mixtures containing human serum samples, the bovine serum level was adjusted such that the total serum concentration of the mixture was 10%.

$CPDG_1$ was purified from an unidentified gram negative soil bacterium at the New England Enzyme Center. The purified enzyme (specific activity 717 units/mg of protein) was sterilized by passage through a 0.45 μm filter and stored at −20°C at a concentration of 535 units/ml. This material was pyrogen-free on animal testing at 125 and 250 units/kg. For treatment, 4000 units (50 units/kg) were injected intravenously over 10 min. Serum $CPDG_1$ activity was measured as previously described [12]. 4-amino 4-deoxy-N^{10}-methyl pteroic acid (AMP) was a gift of Dr. Andre Rosowsky.

RESULTS

Hemodialysis was performed three times on two courses of MTX therapy. The serum MTX concentrations measured before, during and after dialysis are shown in Fig. 1. On none of the three courses did hemodialysis cause a significant drop in the MTX level over and above the expected change due to continuing excretion through poorly functioning kidneys.

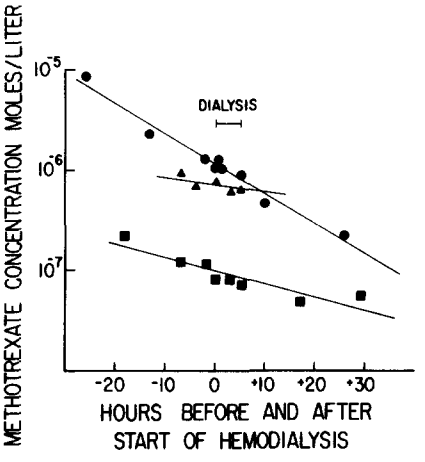

Fig. 1. Change in serum MTX levels during three 5 hr periods of hemodialysis in the same patient. Hemodialysis was performed from hr 50 to 55 (●--●) and 95 to 100 (○--○) after administration of the first course of MTX and from 19 to 24 hr (▲--▲) after injection of MTX on the second course.

Measurements of the MTX clearance across the dialysis coil were made at various flow rates during the first course of dialysis. The data in Table 1 show that the clearance was low at all flow rates tested. Calculations based on the observed clearances indicate that less than 2% of the injected dose was removed by a 5 hr dialysis with a blood flow of 300 ml/min.

$CPDG_1$ cleaves the terminal glutamate from MTX [13], producing AMP which is less cytotoxic than MTX [14]. $CPDG_1$ 50 units/kg was injected intravenously 24 hr after MTX on two courses of therapy. Injection of the enzyme over 10 min caused no change in vital signs and no manifestations of hypersensitivity. The serum MTX levels before and after $CPDG_1$ therapy measured by two different techniques, radioimmunoassay and inhibition of the MTX target enzyme dihydrofolate reductase (DHFR), are shown for both courses in Fig. 2. Within 5 min after completion of the $CPDG_1$ injection, the DHFR kinetic assay detected a 1.0–1.3 log unit decrease in the MTX concentration. Thereafter, serum levels fell with a half-life of 13 and 18 hr on courses one and two respectively. MTX serum concentrations measured by radioimmunoassay showed a different pattern. There was no immediate decrease in MTX

*Obtained from the New England Enzyme Center.

Table 1. *Methotrexate clearance by hemodialysis*

Flow rate*	Arterio-venous difference across kidney†	Clearance‡
100	0.55 μM	0.055
200	0.60 μM	0.12
300	0.13 μM	0.039

*Milliliters/minute.
†Cordis/Dow 1.3 m hollow fiber kidney, model 4.
‡Micromoles/minute.

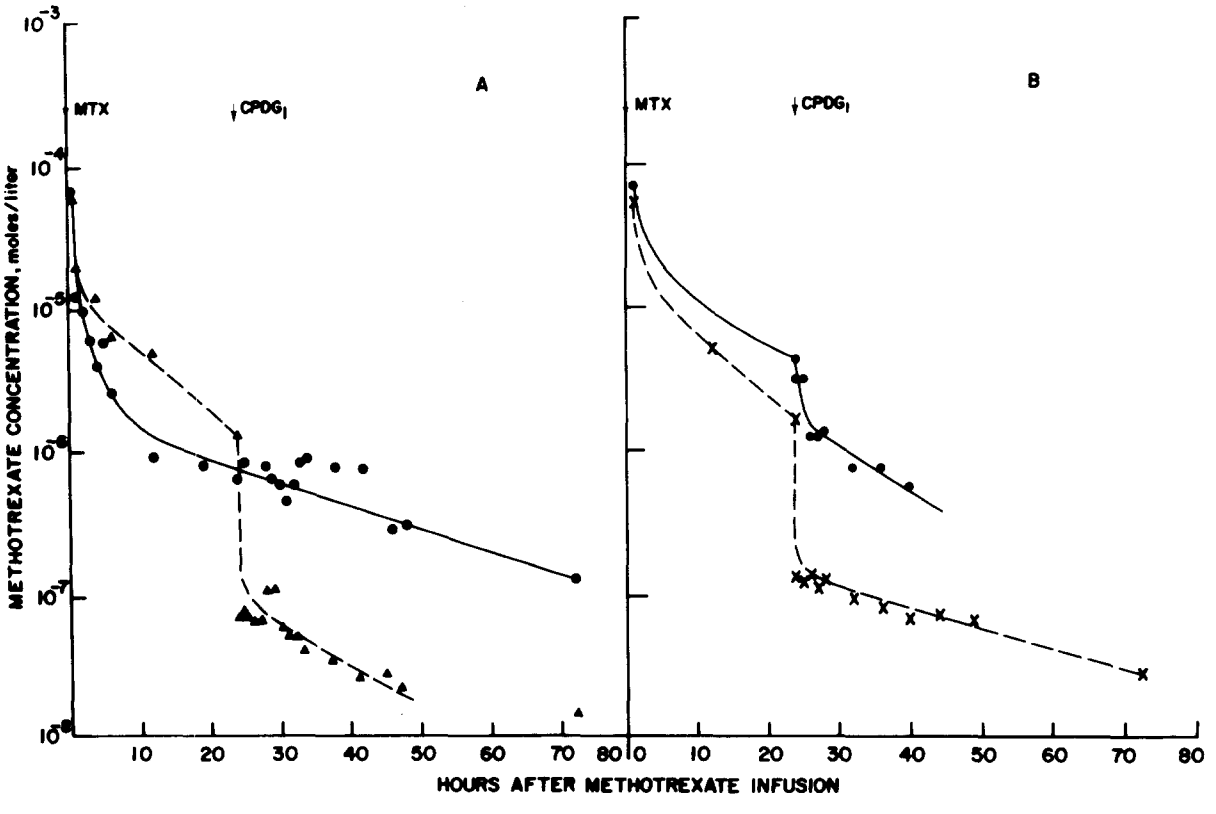

Fig. 2. *Serum MTX concentration measured by radioimmunoassay* (●--●) *and DHFR kinetic assay* (▲--▲) *after rapid infusion of* (*A*) 130 mg/m^2 *and* (*B*) 400 mg/m^2 *in the same patient, CPDG*$_1$ 50 units/kg *was injected* 24 hr *later on both courses.*

levels or changes in the serum half-life on course one and only a minor fall on course two, and following $CPDG_1$ therapy, measurement became erratic. Although no toxicity developed neither course was evaluable for the effect of $CPDG_1$ by itself on preventing clinical MTX toxicity since thymidine rescue was used concomitantly.

Whereas $CPDG_1$ activity was measurable in serum for many hours, the major effect of the enzyme on MTX concentration measured by the DHFR kinetic assay occurred within the first 5 min after enzyme injection. This discrepancy, plus the differences in serum MTX levels detected by the two assays, suggested that the product of the action of $CPDG_1$ on MTX, AMP, may interfere with the ability of both assays to measure MTX. Studies of the relative binding to the antibody currently used in the MTX radioimmunoassay showed equal affinity for MTX and AMP at concentrations between 10^{-5} and 10^{-8} M. Thus any AMP in the serum would be measured by the radioimmunoassay as MTX, which is consistent with the clinical observations.

Figure 3 shows the relative abilities of MTX and AMP to inhibit the DHFR kinetic assay. AMP is 0.29% to 4.6% as effective as MTX across the significant range of the ki-

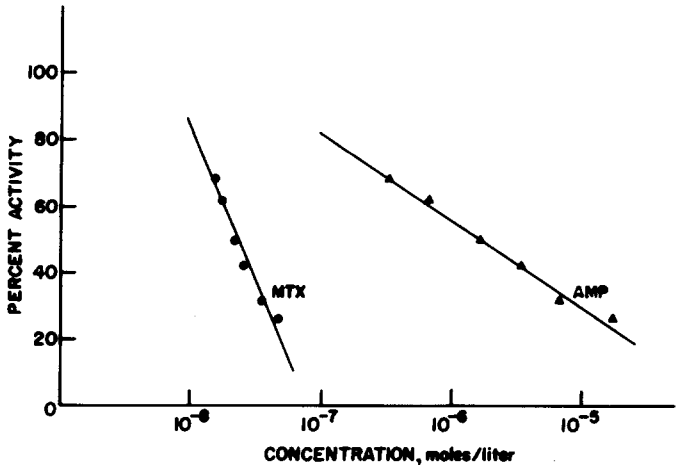

Fig. 3. Inhibition of DHFR activity by MTX and AMP.

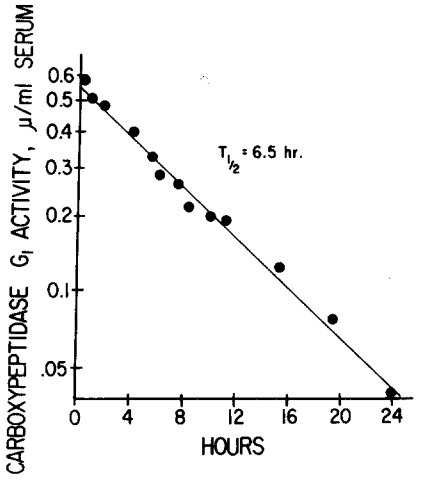

Fig. 4. Serum $CPDG_1$ activity as a function of time after intravenous administration of 50 units/kg *over* 10 min *on the first treatment course.*

netic assay. Thus, if all of the MTX present at the time of $CPDG_1$ injection were converted to AMP, the DHFR kinetic assay would be expected to measure an MTX level of only 1.6 log units lower due to the presence of AMP. This is close to what was actually observed.

Figure 4 shows the measurable enzyme activity as a function of time after injection of $CPDG_1$ on the first course of enzyme administration. The serum half-life of measurable enzyme activity was 6.5 and 4.4 hr on the first and second course of $CPDG_1$ treatment respectively. There was a log-linear fall in serum activity and measurable levels were still present up to 24 hr after injection. The volume of distribution was approximately 6.6 liters, indicating distribution primarily in plasma volume.

DISCUSSION

MTX is a small molecule with a mol. wt of 454. Henderson *et al.* reported that it is 50% bound to serum proteins over the concentration range attained by the high dose MTX/leucovorin program [15]. Based on these properties, MTX ought to be readily removed from blood by hemodialysis. This supposition is supported by the fact that MTX is filtered well across the glomerular basement membrane and that total blood folate levels are reduced by chronic hemodialysis [16, 17]. Several textbooks and reviews have stated that MTX is dialyzable but have not presented any data [18–20]. One brief report indicated that MTX was not readily dialyzable at low serum concentrations [21].

On all three trials in this study, hemodialysis failed to remove significant amounts of MTX. The differences in serum MTX levels before and after the 5 hr dialysis could be accounted for entirely by slow loss of MTX from the blood by other routes. The clearance of MTX by the artificial kidney was found to be low at flow rates of up to 300 ml/min. Extrapolation of the observed clearances indicates that a 5 hr dialysis at optimal flow rates could not have removed more than 6% of the injected MTX dose. In a 70 kg man with a serum MTX level of 1 μM, reduction of the MTX level to a nontoxic range would require removal of more than 95% of the drug. We conclude that hemodialysis is not likely to be useful in the management of patients with toxic levels of MTX and poor renal function. However, more detailed studies are required.

Chabner *et al.* [22] demonstrated that in

mice $CPDG_1$ rapidly reduced MTX levels by more than 10-fold and was as effective a rescue agent as leucovorin. Bertino used $CPDG_1$ (50 units/kg) as a rescue agent five times in three patients, and achieved prompt reduction of serum MTX levels to nontoxic concentrations [23]. The only adverse reaction to $CPDG_1$ observed in man was moderate anaphylaxis associated with the third course of treatment in one of these patients. In the study reported here, two courses of $CPDG_1$ given 13 days apart to the same patient caused no acute or delayed toxicity, and resulted in immediate fall in the MTX concentration of 1.0–1.3 log units measurable by the DHFR kinetic assay.

Several problems were encountered in assessing the results of $CPDG_1$ therapy. $CPDG_1$ activity was detectable up to 24 hr after injection, yet only minor changes occurred in serum MTX levels measured by radioimmunoassay, and what change took place in the MTX levels determined by the DHFR kinetic assay occurred immediately and then appeared to stop. These results can be explained by the following observations. The antibody used in the radioimmunoassay could not distinguish well between MTX and AMP, the product of the action of $CPDG_1$ on MTX. Thus, the radioimmunoassay could not detect conversion of MTX to AMP as shown by the experimental data in Fig. 2. On the other hand, AMP was found to inhibit the DHFR kinetic assay 10-fold less effectively than MTX, which is in agreement with previous reports [14]. Complete conversion of MTX to AMP would thus be expected to cause a 1–2 log unit drop in the MTX level measured by this assay. In fact, on two trials, 1.0 and 1.3 log unit decreases were measured by the DHFR kinetic assay, suggesting that $CPDG_1$ may have converted all the MTX present to AMP.

Enthusiasm for the use of $CPDG_1$ to detoxify MTX must be qualified by two important considerations. First, $CPDG_1$ is immunogenic. Repeated treatment has caused anaphylaxis in one patient [23], and has the potential of further compromising poor renal function by precipitating serum sickness or other pathologic processes associated with circulating antigen-antibody complexes. Secondly, very little is known about the toxicity of AMP. No toxicity was recognized in the 4 patients treated with $CPDG_1$ and limited screening has shown no antitumor effect against murine leukemia [14]. However, AMP is less soluble than MTX and could cause significant crystal-induced nephrotoxicity itself.

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Failure of Lymphocyte Microcytotoxicity to Distinguish Relapsers from Non-Relapsers in Melanoma Patients Receiving Post-Surgical Adjuvant Chemotherapy*

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Abstract—*Our previous studies demonstrated a lack of correlation between sequential lymphocyte reactivity and clinical tumor progression in patients with surgically incurable malignant melanoma. The purpose of the present investigation was to determine whether sequential lymphocyte reactivity correlated with recurrence of disease in melanoma patients with little if any tumor burden. Sixteen stage* I and II *patients with a high risk of recurrence following potentially curative surgery were given adjuvant chemotherapy with dimethyl triazeno imidazole carboxamide (DTIC). Nine patients subsequently relapsed and 7 remained disease free for at least* 44 *months. Each patient was tested for sequential lymphocyte reactivity using cryopreserved lymphocytes and a standard allogeneic melanoma cell line in a microcytotoxicity assay designed to minimize technical variation. No statistically significant difference was found in sequential reactivity between patients who relapsed and those who did not, although lymphocytes from disease free individuals did exhibit a slightly greater increase in reactivity when compared to lymphocytes from patients who recurred. We conclude that the microcytotoxicity assay performed in this way is of little prognostic value in patients with relatively low tumor burdens.*

INTRODUCTION

RECENT publications [1–4] have described a lack of correlation between *in vitro* lymphocyte cytotoxicity assays and clinical tumor progression. However, in only two studies [3, 4] were serial assays performed and in only one [4] was an attempt made to standardize the day-to-day variations in test results. In the present investigation, we tested stage I and II melanoma patients who had definitive surgery (but were at high risk for recurrence) and whose lymphocytes had been cryopreserved throughout the post-operative course. In a retrospective study of sequential lymphocyte reactivity (microcytotoxicity) we attempted to detect a difference in patterns of reactivity between those patients who relapsed and those who remained clinically tumor-free. Thus, we evaluated lymphocyte reactivity serially, in patients with little (if any) residual tumor under circumstances designed to minimize technical variables.

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MATERIALS AND METHODS

Lymphocyte donors

The melanoma patients studied for lymphocyte reactivity (microcytotoxicity) all had local (stage I) or regional (stage II) disease and were all subjected to potentially curative surgery. Entry to the present study was based on a high risk of recurrence: histologically positive regional lymph nodes and/or a primary lesion invasive to Clark's levels III, IV or V [5]. The clinical profile of the study

Table 1. Clinical profile of study patients

		Relapsers/Non-relapsers	
Total patients		9	7
Male/female		7/2	4/3
Age (yr):	Mean	43.8	41
	Median	47	41.1
	Range	21–62	20–60
Primary lesion:			
	Location: trunk	8	6
	extremity	1	1
	Type: SSM	3	5
	NM	4	1
	unclass	1	0
	unknown	1	1
Clark's Level:	III	2	1
	IV	5	4
	V	1	1
	unknown	1	1
Regional lymph nodes:			
	Histologically positive	7	4
	Histologically negative	1	1
	Clinically negative—		
	Histology unknown	1	2
Disease free interval (months):			
	mean	12.8	46.6
	median	12	46
	range	7–20	44+–50+

SSM = superficial spreading melanoma; NM = nodular melanoma; unclass = unclassifiable; III = invasion to the papillary-reticular dermis interface; IV = invasion into the reticular dermis; V = invasion into the subcutaneous fat.

Table 2. General immunologic competence of patients at initiation of study

	Relapsers	Non-relapsers
Total patients	9	7
DNCB positive	7/8*	7/7
≧1 MAST positive	7/8*	7/7
Mean No. MAST positive	2.1	1.7
IgG	7(N), 1(↑), 1(↓)	7(N), 0(↑), 0(↓)
IgA	6(N), 2(↑), 1(↓)	3(N), 3(↑), 1(↓)
IgM	9(N), 0(↑), 0(↓)	5(N), 2(↑), 0(↓)

DNCB = dinitrochlorobenzene; MAST = microbial antigen skin tests (purified protein derivitive, mumps, candida, tricophyton and coccidioidin), Ig = immunoglobulin; N = normal; ↑ = increase; ↓ = decrease; * = one patient not tested.

patients is presented in Table 1. Of interest, 11 of 16 patients had histologically positive regional lymph nodes. The general immunologic profile of the melanoma patients is presented in Table 2. As assessed by these parameters, the study patients were immunologically competent.

Between 4 and 8 weeks following definitive surgery, all patients were started on adjuvant chemotherapy with dimethyl triazeno imidazole carboxamide (DTIC): 2 mg/kg/d × 10 days (IV), every three months × 5. Blood samples for lymphocytes and serum were collected immediately prior to the initiation of each cycle of chemotherapy and every three months following completion of chemotherapy until relapse or for 20–22 months in patients who remained disease free. Non-relapsers were defined as those patients who remained disease free for at least 44 months after surgery and relapsers were those patients found to recur no sooner than 6 months after surgery.

Microcytotoxicity assay

Lymphocytes were separated from heparinized blood on a Ficoll–Hypaque gradient and stored in liquid nitrogen as described previously [6]. Blood from melanoma patients was allowed to clot at room temperature. The sera were separated by centrifugation, and stored at −20°C. Immediately prior to use, the sera were heat inactivated at 56°C for 30 min. A single melanoma cell line (M14), pooled and stored in liquid nitrogen as previously described [6], was used as the target cell throughout this study.

Our modification of the Takasugi and Klein [7] microcytotoxicity technique has been described in detail elsewhere [6]. Each experimental and control group consisted of 8 replicate wells. Prior to addition of lymphocytes, either heat inactivated fetal calf serum (FCS) diluted 1/6 with Prehn's modification of Eagle's medium (PEM; Associated Biomedic Systems, Inc., Buffalo, New York), or serum autologous to the lymphocytes being tested (also diluted 1/6 with PEM), was added to the target cells in 0.01 ml amounts. Patient's lymphocytes in PEM containing 20% FCS were then added to the target cells (300 lymphocytes/target cell) and the mixture incubated for 48 hr. The remaining melanoma cells were stained and counted as previously described [6]. To minimize test variation, pretreatment and post-treatment lymphocyte samples from a single patient were on the same plate. In addition, on each plate there were 8 wells containing target cells plus FCS diluted 1/6 with PEM.

Criteria for analysis

Changes in lymphocyte reactivity after surgery were determined by linear regression analyses. Student's *t*-tests were calculated to compare the slopes obtained for each patient in the presence of FCS and in the presence of patient's serum. Several comparisons of these slopes were made by the standard analysis of variance. Relapsers were compared with non-relapsers for reactivity both in the presence of fetal calf serum and in the presence of patient's serum.

RESULTS

To determine the change in reactivity, lymphocyte samples from each of the 16 melanoma patients studied were retrieved from liquid nitrogen and tested simultaneoulsy against allogeneic melanoma cells (M14) as described in Materials and Methods. All the lymphocyte samples collected during the 20–22 month period following surgery were tested for the non-relapsers (Fig. 1), while those of relapsers (Fig. 2) were tested up to the date of clinical recurrence.

Student's *t*-tests indicated that for each patient there was no significant difference between the slope of lymphocyte reactivity in the presence of FCS and in the presence of

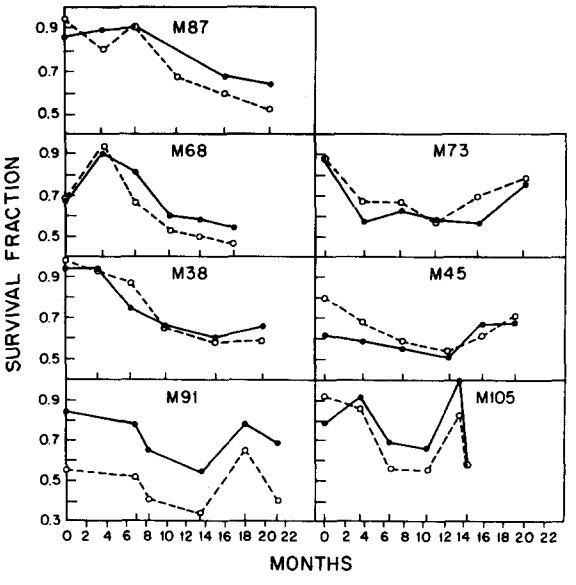

Fig. 1. Sequential reactivity against M14 melanoma cells of lymphocytes plus patient's serum (——) and lymphocytes plus fetal calf serum (– – –) from 7 stage I or II melanoma patients who were disease free ≧44 months following definitive surgical therapy. Survival fraction was calculated by dividing the number of tumor cells in wells containing lymphocytes plus serum by the number of tumor cells in the wells containing medium alone.

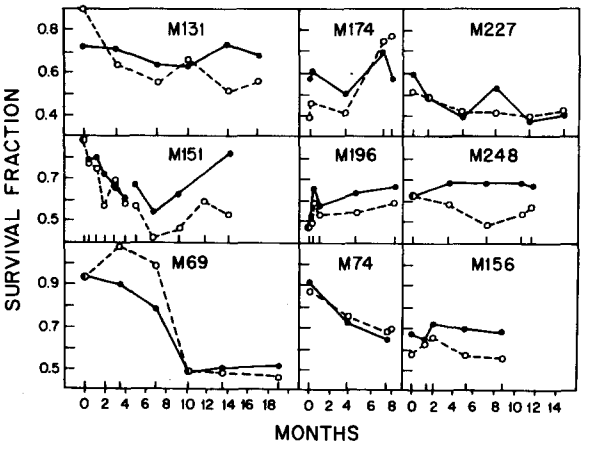

Fig. 2. Sequential reactivity against M14 melanoma cells of lymphocytes plus patient's serum (——) and lymphocytes plus fetal calf serum (– – –) from 9 stage I or II melanoma patients who relapsed between 7 and 20 months following definitive surgical therapy. Survival fraction was calculated by dividing the number of tumor cells in wells containing lymphocytes plus serum by the number of tumor cells in wells containing medium alone.

patient's serum, although the slope in the presence of FCS was often steeper.

Comparisons between relapsers and non-relapsers by the standard analysis of variance indicated no significant difference in slopes, although both in the presence of FCS and in the presence of patient's serum the average of the slopes was steeper for non-relapsers than for relapsers.

Linear regression analyses were also run after pooling data for each group and ignoring differences among patients. Again the slopes were not significantly different from each other, although again the slopes for the non-relapsers tended to be a bit steeper than those of the relapsers.

DISCUSSION

Several investigators have attempted to correlate the results of a single lymphocyte microcytotoxicity (LMC) assay with prognosis. Veronesi *et al.* [1] studied 18 stage I and II melanoma patients who had been subjected to potentially curative surgery. Seven of 12 LMC-positive patients relapsed (mean 8.4 months) as did 5/6 LMC-negative patients (mean 8.0 months) (P=N.S.). Nairn *et al.* [2] were also unable to correlate results of LMC testing with prognosis; 3/5 LMC-positive patients recurred. (The relapse rate for LMC-negative patients was not reported.) A firm conclusion regarding the prognostic value of a single LMC assay cannot be drawn from these preliminary data. Only a modest number of patients have had LMC measured serially. Hellström *et al.* [3] performed sequential studies of LMC in 10 melanoma patients. LMC was present in patients who were tumor-free or who had small tumor burdens as well as in patients with larger tumor burden. The data suggested that patients with more modest tumor burdens had more intense LMC than did patients with larger tumor burdens. In individual patients, LMC varied widely with time and was not useful prognostically. Pavie-Fisher *et al.* [4] used frozen lymphocytes and two long-term target cell lines in their serial studies of 25 patients (8 tumor bearing) for up to 10 months. In only 12/25 patients was LMC noted at least once. Despite this attempt at standardization of test conditions, wide variation in results was noted and there was no correlation with prognosis. The authors suggested that the variable cytotoxic capacities of control lymphocytes may have been a contributory factor. Heppner *et al.* [8] performed multiple LMC assays on a number of patients but these generally spanned a short time interval and no effort was made to correlate the LMC assay data with prognosis.

These studies may have failed to demonstrate a positive correlation between the results of the LMC assay and prognosis for a variety of clinical and technical reasons. Few patients have been studied serially through a protracted disease free interval to recurrence. Even when serial studies are performed, day to day variations in assay conditions make comparisons of data points on the same patient difficult. The important technical variables have been previously discussed in detail [9] and include: the target cell, effector cell to target cell ratio, inconsistent cytotoxicity of control lymphocytes, and duration of incubation. In the present study, an attempt was made to minimize these technical variables. One subculture of a single allogeneic melanoma cell target was used throughout. The effector cell to target cell ratio was constant. Medium and not "normal" lymphocytes was used as a control. Lymphocytes were cryopreserved and all samples on a single patient were tested on the same day and on the same plate. Further serial sampling over time allowed each patient to serve as his own control. In order to insure a homogeneous study population, with, at most, micrometastatic disease, patients who relapsed in less than six months following surgery were excluded. Despite these precautions, the present study failed to demonstrate a correlation between LMC data and prognosis.

The inability to distinguish between patients who relapsed and those who remained disease free could have been a result of several other factors. First there was a suggestion, although not significant, that the non-relapsers increased in lymphocyte reactivity to a greater degree than relapsers. A greater difference might have been masked by the chemotherapy. Since postsurgical patients receiving no treatment were not available for study, the effect of the DTIC treatment could not be determined. However, we were unable to demonstrate a significant difference in pre- and post-DTIC treatment LMC assays in patients bearing clinically evident melanoma [10]. Secondly, some of the non-relapsers may recur at a later date and thus were wrongly classified. To minimize this possibility, only patients who were disease free for a prolonged period (>44 months) were used as controls. The median disease free interval for relapsers was 12 months compared with 46 months for non-relapsers. Finally, despite indications of a

common melanoma antigen [9], allogeneic systems may not be appropriate for measuring tumor immunity. The use of autologous tumors in this study would have been completely impractical because of the numbers of patients involved.

Serum blocking activity may correlate more closely with prognosis than the LMC assay [3, 9]. Autologous serum effects were assessed serially in the present study and failed to distinguish those patients who relapsed from those who remained disease free. However, the conventional calculation of serum blocking effect (i.e. target cell survival in the presence of patient's lymphocytes and patient's serum minus target cell survival in the presence of patient's lymphocytes and control serum) was not attempted because the tests with patient's serum were not performed on the same plates as the tests with control serum.

In summary, it appears that the lymphocyte microcytotoxicity assay, as performed here, does not correlate with melanoma progression.

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Ewing's Sarcoma, Adjuvant Chemotherapy and Pathologic Fracture

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Abstract—*Between* 1970 *and* 1975, 19 *patients under age* 15 *with Ewing's sarcoma have been treated with adjuvant chemotherapy in addition to radiotherapy to the primary tumour. The chemotherapy regimen of vincristine and cyclophosphamide was not rigorously structured and evolved over the six years. The two most recent patients have also had adriamycin. The median survival of* 45 *months is statistically better than the* 18 *months experienced by the* 31 *children treated without adjuvant chemotherapy from* 1956 *to* 1969. *Eight pathologic fractures have occurred in* 6 *of the* 19 *patients on adjuvant chemotherapy. Only one of the fractures was at diagnosis. The other* 7 *fractures occurred from* 3 *to* 72 *months following diagnosis with a median of* 6 *months. There were only* 3 *pathologic fractures in the* 31 *patients treated without adjuvant chemotherapy—two were at diagnosis and one was at a metastatic site. The increased risk of pathologic fractures appears to be related to the adjuvant chemotherapy.*

INTRODUCTION

THE EFFECTIVENESS of chemotherapy for microscopic metastases in Ewing's sarcoma [1, 2] has been supported by the recent experience (1970–1975) at the Christie Hospital and Holt Radium Institute and the Royal Manchester Children's Hospital. Analysis of the results of the patients treated with adjuvant chemotherapy suggest an increased risk of pathologic fractures unrelated to simple increased length of survival. In this era of chemotherapy which has generally increased the rate of cure in most childhood sarcomas and embryonal tumours the risk of therapy and quality of survival need to be observed closely. Children who are cured may well carry the side effects of therapy for many decades. As therapy regimens are refined, hopefully cure rates can be increased while reducing both short- and long-term morbidity.

MATERIALS AND METHODS

By 1970 the Christie Hospital and the Royal Manchester Children's Hospital had become the established centre in a large area of Northwest England for the care of children under 15 with malignancies. The Manchester Children's Tumour Registry's case finding method as well as pathology review system has been previously described [3, 4]. The registry shows that all patients in the geographical area from 1970 had been referred to the Christie Hospital following diagnosis and it also served as the source for the control group of children treated before 1970. The diagnosis of Ewing's sarcoma was accepted as such if a child was so listed in the Children's Tumour Registry.

The chemotherapy plan for the period 1970–1975 was not rigorously structured and evolved over the 6 yr of the study. However, the basic concept of frequent doses at moderate levels of vincristine and cyclophosphamide for at least one year remained consistent throughout the study. A total of 19 patients, 14 of whom had no metastases at diagnosis, were treated during the 6 yr period (1970–1975). Therapy evolved through three phases. The first 11 patients received vincristine 1.5 mg/M^2 alternating with cyclophosphamide 300–400 mg/M^2 weekly for 6 months, then the two drugs were given together every two weeks for 6–12 months more. The chemotherapy plan for the next 6 patients was changed so that the two drugs were given initially, in approximately the same doses as before, every two weeks together for 6 months, and then every month for one further year. Finally, because of very favourable phase two data [5] adriamycin has been

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added to the regimen of the most recent 2 patients. The chemotherapy programme remains as for the previous six patients, except that a single dose of adriamycin 40 mg/M^2 is given in addition every 6 weeks for 1 yr.

The radiation therapy has remained consistent for all patients since 1956. The radiation has been delivered to the whole affected bone, to a dose of 4000–4500 in three weeks at 4 MeV. It has not been possible to achieve this dose and fractionation for all of the flat bone lesions.

The control group consists of 31 patients treated from 1956 through 1969. Three of these 31 had amputations of the involved bone as primary therapy while none of the 19 patients since 1970 had an amputation as primary therapy. The age, sex and primary tumour sites of the two groups is delineated in Table 1. Evaluation of the patients treated before 1970 following their irradiation was not regimented though they usually had both chest and local lesion diagnostic X-rays every 1–3 months for the first year, and slightly less frequently during the second year. Since 1970 the patients have had diagnostic X-rays of the chest and local lesion routinely at least every one to two months for the first year and then every three months for the next 2 yrs. Other diagnostic tests were done as clinically indicated.

Table 1. *Ewing's sarcoma, Manchester* 1956–1974. *Age, sex and site of primary tumour*

	Control	Chemotherapy
No. of patients	31	19
Age range	2–14	<1–14
Median	8	11
Sex: male	17	12
female	14	7
Primary site		
Upper extremity	7	6
Lower extremity	15	5
Pelvis	5	4
Other	4	4

Statistical note

Curves for survival, local recurrence, metastases and pathological fracture were calculated by the product-limit method [6], and statistical significance of differences between groups of patients was analysed by the log-rank method [7].

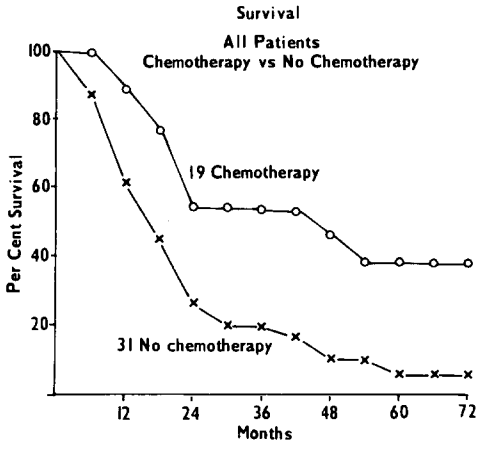

Fig. 1.

RESULTS

Survival

Of the 31 patients treated before 1970 only 2 survive while 11 of the 19 treated since 1970 are alive (Fig. 1). Of these 11 survivors, 3 are alive with disease. The median survival for the patients treated without chemotherapy was 18 months while it was 45 months for all patients treated with chemotherapy. For the 14 patients who had no evidence of metastases at time of diagnosis and treated with chemotherapy, seven are alive and disease-free for 10, 17, 22, 22, 63, 68 and 69 months (Fig. 2). One patient with a primary tumour in the distal femur, a positive groin node by biopsy and an abnormal lymphangiogram at diagnosis, is alive over 6 yr and has been off all therapy almost 5 yr. The difference in survival for the total group treated before chemotherapy compared to the group of 19 treated with chemotherapy is significant at the $P<0.01$.

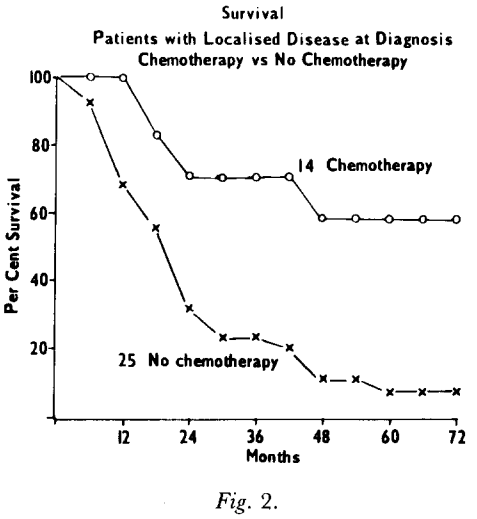

Fig. 2.

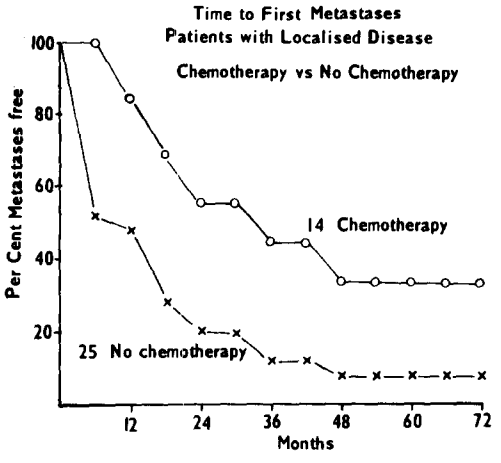

Fig. 3.

Metastases

As can be seen in Fig. 3, the rate of metastasis is greatly reduced in those patients who were disease-free at diagnosis and treated with adjuvant chemotherapy. The difference is significant at the 1% level. In the chemotherapy group, metastases did develop as late as 46 months from diagnosis though late metastases also occurred in the non-chemotherapy group.

Local recurrence

The 19 patients who were treated with chemotherapy had 3 local recurrences compared to 9 local recurrences in the 31 patients treated before 1970. Another patient on chemotherapy had radiographic changes consistent with a local recurrence five months following radiotherapy. An amputation was done, but no viable tumour was found and he remains well 5 yr later. The 3 local recurrences in patients treated with chemotherapy were at 13, 14 and 34 months. In the pre-chemotherapy group the local recurrences were diagnosed from 3 to 31 months from initial diagnosis and in 2 of these 9 the local recurrences were noted after there was evidence of distant spread. Analysis suggests that the frequency of local recurrence has been reduced by the chemotherapy though the P value (0.056) is not quite small enough for statistical significance.

Complications

Two patients in the pre-chemotherapy group developed significant complications. In one child diagnosed with a tibial Ewing's sarcoma at age 3, deformity secondary to the radiotherapy led to an amputation at age 7. She remains well 18 yr from diagnosis. In a second child who was 13 at diagnosis of her tibial Ewing's sarcoma, a local recurrence 17 months later was treated with local excision. However, she required an amputation due to poor local healing following this second procedure.

The chemotherapy was very well tolerated. There were no episodes of severe bone marrow depression and no episodes of sepsis or local infection. Reduction of the dose of cyclophosphamide or lengthening of the interval between doses was rarely indicated. There were two episodes of haemorrhagic cystitis. One occurred after 4 months of therapy and another after 12 months. Cyclophosphamide was discontinued in both, and both patients developed pulmonary metastases within 4 months and died.

Six of the nineteen patients treated with chemotherapy have developed 8 pathologic fractures at the primary tumour site. (Table 2). Only one of these fractures was at diagnosis and this patient has had a second pathologic fracture at 68 months from diagnosis. The other patient to develop two pathologic

Table 2. *Pathologic fractures in patients treated with adjuvant chemotherapy*

Patient	Primary site	Months from diagnosis of pathologic fracture	Results
WD	Distal femur	0	Union
		68	Immobilized
ND	Proximal tibia	8	Union
		72	Immobilized
GP	Proximal femur	3	Non-union
AF	Proximal humerus	4	Union
PF	Proximal humerus	6	Union—24 months later X-ray evidence of recurrence. Amputation. No tumour microscopically
JB	Pubis	6	Union—local recurrence 8 months later

fractures had his first fracture at 8 months and his second at 72 months following diagnosis. The median time to pathologic fracture was 6 months. Of the 8 fractures, 5 have healed following immobilization, two remain immobilized and one is a non-union. In only 1 of the 6 has a local recurrence been diagnosed and that occurrrd 8 months following fracture. Two patients have had fractures in upper extremities and 3 patients have had 5 fractures in lower extremities. It is also of interest that a 14-yr-old girl who was initially diagnosed as having a Ewing's sarcoma of the right eighth rib and treated accordingly with radiation and vincristine and cyclophosphamide, had her diagnosis eventually changed to undifferentiated sarcoma and is not included in this analysis. However, she developed a pathologic fracture 4 yr after diagnosis and it has failed to unite.

In contrast only 3 of the 31 patients treated before 1970 have developed pathologic fractures—two were at the primary site at the time of diagnosis and one was at a metastatic site. This increase does not appear to be due to greater length of survival in the chemotherapy group, but apparently to other factors since most pathologic fractures in the chemotherapy group were before the median survival time of the control group.

DISCUSSION

These results are consistent with other reports that adjuvant chemotherapy in Ewing's sarcoma markedly increases the length of disease-free survival and that it apparently improves the cure rate [8]. This series like all others to date is too small, considering all the prognostic variables, to determine which therapy regimen is most effective [9]. Programmes using similar therapy have produced comparable results and have been reviewed [8]. The report from Memorial Hospital, where a much more aggressive regimen for both radiotherapy and chemotherapy was used, appears to have better initial survival rates [10].

Two aspects of this study show very important changes produced by chemotherapy. Reviews of radiotherapy doses to sterilize the primary site have shown a plateau effect somewhere around 4500–5000 rad [11, 12]. Local recurrence rates have tended to be about 25% even in lesions irradiated above 6000 rad. However, in this series the rate of local recurrence tends to be lower in those patients who have received chemotherapy. This suggests that the chemotherapy is able to affect the local tumour, as suggested by Fernandez [12]. Why increasing doses of irradiation are unable to sterilize tumours that responded initially, is problematic. However, the prolonged courses of cytotoxic therapy may well be able to penetrate revascularized bone or affect cells when they come out of a resting phase. Increasingly higher doses of irradiation may only serve to increase local morbidity of both the bone and soft tissues, especially in growing children [13].

The high incidence of pathologic fractures appears to be a new problem. The occurrence of these fractures seems to be unrelated to length of survival, weight bearing or significant local trauma. None of the 8 fractures occurred after significant trauma and they occurred in both upper and lower extremities. This increase must be related to the addition of chemotherapy since the radiotherapy regimen at this institution has not changed during the time of the study. Hustu, Pinkel and Pratt [14] mention that 2 of their 15 patients also sustained pathologic fractures. Pathologic fractures of the pelvis and neck of the femur and of the humerus following radiation for uterine and breast tumours respectively, have been well documented [15, 16]. The radiographic bone changes produced following different energy irradiation has recently been well described [17].

The incidence of fractures in our patients is extremely high though all were treated with 4 MeV irradiation which is apparently less damaging to bone than lower energy irradiation. The radiation dose used is significantly lower than that of many other centres. Howland *et al.* [17] described a latent period for fractures whose mean is 3 yr. The short latent period in our group suggests added insults from the primary lesions as well as from the chemotherapy. The chemotherapy may act as a radiomimetic agent and it may also affect bone healing. Little is known about the effects on bone of cyclophosphamide and vincristine though cyclophosphamide can affect the development of ossification centres in foetal rats [18]. Another chemotherapeutic agent, methotrexate, is known to affect bone metabolism [19] but the mechanism of action of this agent is quite different from those of vincristine and cyclophosphamide. Pathologic fractures may become a major form of long-term morbidity in these patients.

The therapy of pathologic fractures under these circumstances remains to be evaluated. One of Hustu's *et al.* [14] two fractures became a non-union. Two of our fractures are too recent to speculate on the outcome. Boyes (1974) [20] reported a woman who, 6 months after diagnosis of a reticulum cell sarcoma of the femur, developed a pathologic fracture. Several types of internal fixation, besides bone grafts, were used to gain stabilization. The non-union persisted for 12 yr, but the internal fixation had stabilized the bone.

The use of operative therapy for these fractures should probably remain a last resort to be explored. How well damaged bone and skin will tolerate internal appliances as well as heal, needs to be evaluated.

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Europ. J. Cancer Vol. 14, p. 805. Pergamon Press 1978. Printed in Great Britain

Addendum

The article by P. Alberto, M. Rozencweig, D. Gangji, A. Brugarolas, F. Cavalli, P. Siegenthaler, H. H. Hansen and R. Sylvester, entitled "Phase II study of anhydro-ara-5-fluorocytidine in adenocarcinoma of gastrointestinal tract, epidermoid carcinoma of lung, head and neck, breast carcinoma and small cell anaplastic carcinoma of lung", published in Volume 14, p. 195 (February 1978), was also supported by Contract NO1/CM/53840 of the National Cancer Institute, Bethesda, Maryland, U.S.A.

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Announcements

CANCER OF THE GENITOURINARY TRACT

"Cancer of the Genitourinary Tract" will be the topic of the 23rd annual Clinical Conference to be held in Houston at the Shamrock Hilton Hotel, 2–3 November, 1978. The conference will focus on alternatives in therapy for urological malignancies. Co-chairmen: Dr. Douglas E. Johnson and Dr. Melvin L. Samuels.

For additional information: Stephen C. Stuyck, Information Coordinator, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030.

INTERNATIONAL SYMPOSIUM ON THE DIAGNOSIS OF LIVER METASTASES

The E.O.R.T.C. (European Organization for Research on Treatment of Cancer) is organizing an International Symposium on The Diagnosis of Liver Metastases which will take place in Brussels on 30 November–2 December, 1978 at the Institute of Sociology of the University of Brussels (U.L.B.).

The Symposium will analyse the various diagnostic methods currently used and their optimal combination for the detection of liver metastases. The technics concerned are: Ultrasonography, Radionuclide imaging (Scintigraphy), Computed Tomography (CT-Scan) and Peritoneoscopy.

The Organizing Committee: H. Bleiberg, J. Frühling and M. Osteaux, Jules Bordet Institute, 1, rue Héger Bordet, 1000 Brussels, Belgium

FOURTH CONGRESS OF THE MEDICAL ONCOLOGY SOCIETY, NICE, FRANCE

2, 3 and 4 December, 1978

Direct inquiries to: Pr. M. Schneider, Centre Antoine-Lacassagne, 36, Voie Romaine, 06054 Nice, Cedex, France.

SECOND INTERNATIONAL CONFERENCE ON THE ADJUVANT THERAPY OF CANCER

The Second International Conference on the Adjuvant Therapy of Cancer will be held in Tucson, Arizona, 28–31 March, 1979, under the sponsorship of the Cancer Center Division of the University of Arizona. Drs. Sydney E. Salmon and Stephen E. Jones will serve as Conference Co-Chairmen. The deadline for submission of abstracts (prepared in the format of AACR/ASCO) is 1 December, 1978. For further information, write to:
Ellen Gerrity,
Conference Coordinator,
Cancer Center Division,
University of Arizona,
Tucson, Arizona 85724.

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Early Polykaryocytosis Inhibition: A Simple Quantitative *in vitro* Assay for the Detection of Bovine Leukemia Virus Infection in Cattle*

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Abstract—*Extensive early polykaryocytosis was consistently observed in mixed cultures of bovine leukemia virus (BLV) infected ovine cells and mouse sarcoma virus infected cat cells. It is shown that syncytia are induced by the BLV infected cells. There is a quantitative, but non linear relationship, between the amount of BLV infected cells and the syncytia response. Polykaryocytosis is at least partially mediated by complete leukemia virus or by some of the envelope and internal antigens. The reaction is specifically inhibited by bovine sera known to contain antibodies directed against BLV, but not by normal sera.*

Quantitative studies permitted to develop a simple, rapid and reliable in vitro *assay aimed at the detection of BLV related antibodies (EPI). The methodology to be applied for the analysis of the results and for the comparison of the potencies of different sera is described.*

A preliminary application of the assay to the survey of BLV infection in cattle gave results in accordance with those obtained in a parallel survey performed with the hematological and the complement fixation techniques. However, a higher sensitivity was found in the former. In addition, the sera of calves with lymphosarcoma and one case of cutaneous lymphosarcoma were found to react positively in the EPI assay suggesting that BLV genes are also involved in the sporadic forms of the disease.

INTRODUCTION

SYNCYTIAL formation was described by Cornefert-Jensen and her collaborators [1] in cocultures of certain cell lines with leukemic bovine lymphoid cells. They later found that sera from cattle with lymphosarcoma inhibited the formation of syncytia [2]. Although they referred to the causative agent as bovine syncytial virus, it was morphologically like a C-type virus and unlike the foamy-like bovine syncytial virus (BSV) described by Malmquist *et al.* [3]. A bovine C-type (leukemia) virus (BLV) released by cultured lymphocytes was described [4] and further shown to be involved in cattle leukemogenesis. Large scale surveys aimed at the detection of BLV infection in cattle using immunofluorescence [5, 6], immunodiffusion [7] and complement fixation [8] techniques gave results comparable to those previously obtained by Cornerfert-Jensen *et al.* [2].

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The syncytia inducing ability of bovine leukemic cells was further investigated by Diglio and Ferrer [9] who found that this property could be attributed to BLV itself. They thus proposed a procedure for the titration of virus-neutralizing antibodies [10]. The syncytia that they described appear after a relatively long incubation period and the results of assays were evaluated after 4–8 days.

In addition to this cytopathogenic effect, Irgens *et al.* [11] found that syncytia were repeatedly obtained when the BLV infected fetal lamb kidney cell line (FLK-BLV) established by van der Maaten and Miller [12] was cocultivated with the murine sarcoma virus infected cat cell line (CC 81) prepared by Fischinger and his associates [13]. These syncytia were very abundant, with a maximum amount observed as soon as 24 hr after initiation of the cocultures. In addition, their size was most often considerably larger than previously reported. These observations prompted us to further investigate this phenomenon.

In this report we demonstrate the following: syncytia are induced by the BLV infected cells; there is a quantitative, but non linear relationship, between the number of BLV infected cells and the syncytia response; polykaryocytosis is at least partially mediated by complete bovine leukemia virus or by some of the envelope and internal antigens; the finding that the reaction is specifically inhibited by bovine sera, known to contain antibodies directed against BLV, but not by normal sera permitted us to develop a simple *in vitro* early polykaryocytosis inhibition (EPI) test, the results of which are obtained in 24 hr.

The EPI assay and the methodology to be applied for the analysis of the results and for the comparison of the potencies of different sera is described in this study. A preliminary application to the survey of BLV infection in cattle is also reported.

MATERIALS AND METHODS

Animals and sera

The hematological status of all animals studied was determined according to the leukosis evaluation guide of Bendixen [14].

Cow 333, a case of persistent lymphocytosis (LP), is maintained in our laboratory facilities. Its hematological status and virus production in cultures of buffy coat cells were previously described [15]. Serum 1121 of this cow has complement fixing antibodies to BLV antigens [16, 17]. Several samples of the serum were extensively used as reference in the studies on syncytia inhibition. Cow LB 44 was a referral case of adult lymphosarcoma (LSA); buffy coat cells of this animal release abundant C-type virus particles upon cultivation and the serum contains complement fixing antibodies. The control normal cow (H) kept in our facilities for several years has a normal blood picture and all the above tests were negative.

Serum 647 is from a cow with LP and was used directly or after centrifugation (50,000 ***g***, 1 hr) a procedure which increases the potency of the sera in EPI assays. Sera 1 to 11, kindly referred to us by Dr. C. Olson, were described elsewhere [18]. Serum 461 is from a cow with no hematological and immunological signs of leukemia or lymphocytosis and which belongs to a leukemia-free herd.

The other sera of cows with adult lymphosarcomas (LSA), juvenile lymphosarcomas (LSJ) or cutaneous lymphosarcoma (LSC) were from referral cases. Those from clinically and hematologically normal contact animals (NC) and from cows with LP were found in an epidemiological survey. The sera from normal cattle (N) were randomly selected in leukemia-free herds. All sera were heat inactivated (56°C, 10 min) prior to use in early polykaryocytosis inhibition assays.

Anti foamy-like bovine syncytial virus (BSV) serum 449 was generously supplied by Dr. J. F. Ferrer (School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania). This serum was obtained from a cow in the BLV-free study herd BH [10] and is negative for BLV antibodies.

Cell cultures

Fetal lamb kidney cells chronically infected with the bovine leukemia virus (FLK-BLV) were kindly supplied by Dr. M. J. van der Maaten and Dr. J. M. Miller [12] (National Animal Disease Laboratory, U.S. Department of Agriculture, Ames, Iowa). Electron microscopic observation of these cells revealed abundant C-type virus production and no apparent cell degeneration associated with virus release. There was no evidence of mycoplasma or of other viruses common in cattle. The virus found in FLK-BLV cell cultures was indistinguishable by immunological and biochemical means from the virus obtained from cultures of bovine leukemic or lymphocytotic lymphocytes [12, 19].

The murine sarcoma virus infected cat cell line, CC 81 [13] was a gift of Dr. Péries (Institut de Recherche sur les Leucémies et les Maladies du Sang, Laboratoire d'Hématologie Expérimentale, Hôpital Saint-Louis, Paris, France).

The above cell lines were maintained as monolayers in RPMI 1629 culture medium supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml fungizone.

Large amounts of lymphoid cells were obtained either by continuous flow centrifugation of whole blood as described elsewhere [17], or by the improved ammonium chloride solution method of Odajima and Sonoda [20]. Stationary cultures were prepared in RPMI 1640 spinner modified culture medium containing the same additives as for monolayer cell cultures.

BLV concentration and purification

The culture fluid used for the growth of FLK-BLV cells was the *in vitro* source of BLV. After differential centrifugation, the pellet was resuspended in buffer (Na_2HPO_4-citrate 0.02 M pH 7, NaCl 0.1 m, EDTA 0.001 M) and subjected to isopycnic centrifugation in preformed gradient of 20–50% sucrose in the same buffer (95,000 ***g***, 16 hr).

BLV antigens

All preparations of BLV antigens were made from virus purified by gradient centrifugation. For BLV internal antigens, 20 vol of peroxide-free ether were mixed with the BLV suspension and continuously agitated for one hour. Ether was then eliminated by evaporation in nitrogen. The aqueous phase was collected and the soluble internal antigens were clarified by centrifugation (100,000 ***g***, 1 hr).

In order to expose virion surface antigens more specifically, a method previously described (17) was employed. In brief, suspended purified BLV was fixed with 1.2% glutaraldehyde for 3 hr at 4°C. After dilution of the treated BLV preparation in phosphate buffered saline (PBS) and centrifugation (50,000 ***g***, 2 hr) the sedimented material was resuspended in a small volume of the same buffer and used as antigen. A control preparation was made in the same manner after concentration and purification of an equivalent amount of mouse radiation leukemia virus complex, Duplan isolate (RadLV-D) produced by the 13-3-C cell line [21].

Absorption experiments

To absorb with viral preparations, 0.125 ml reference serum 1121 was preincubated with an equal volume of virus internal antigen or glutaraldehyde-treated virus preparations. In some cases, the same volume of serum 1121 was mixed with 0.0625 ml virus internal antigen and the same volume of glutaraldehyde-treated virus preparations. After 1 hr incubation (37°C), the residual titer of the reference serum was estimated by the syncytia inhibition assay. The culture fluid of bovine cell cultures was also used as antigen. For this, 0.25 ml serum 1121 was mixed with 5 ml culture fluid. After incubation (1 hr, 37°C) the volume was brought back to 0.25 ml by Minicon B 15 concentration and the titer of the serum estimated as above.

To absorb with cells, FLK-BLV cells in culture and bovine leukocytes either cultured for 48 hr or non cultured were suspended in 0.25 ml PBS and incubated with an equal volume of serum 1121 for 1 hr at 37°C. After centrifugation (4000 ***g***, 10 min) the titer of the serum was determined.

Complement fixation technique

The technique described by Miller and van der Maaten [8] was used after modification as previously reported [16].

Syncytia assay

FLK-BLV and CC 81 cells, in culture medium supplemented with five times the usual amount of penicillin and streptomycin, were seeded at the desired combinations of concentrations into the wells of Falcon Microtest II tissue culture plates. These were then tightly closed with a sealing tape and placed in an ordinary incubator (37°C) for 14–24 hr. After washing three times with PBS, the monolayers were fixed with methanol (70%) and stained with Giemsa. The presence and number of syncytia per well were monitored with an inverted microscope (magnification 60) by examining the whole surface or only 8 mm^2, depending on syncytia concentration. Counts were performed at least in duplicate wells.

Early polykaryocytosis inhibition (EPI) assay

Two procedures were used for serum titrations; they are detailed in Table 1 and the

rational of the different steps explained in section "Results". The complete procedure requires careful counting of syncytia in FLK-BLV and CC 81 cell cocultures performed in Falcon Microtest II tissue culture plates, whereas the simplified EPI assay needs only an approximate overall eye estimation. In both cases, results may be obtained 24 hr after planting FLK-BLV cells. The titers of the sera are expressed as the reciprocal of the dilution which gives a 50% reduction of the number of syncytia as compared to the non treated controls cultures (50% syncytia inhibitory dose or SID_{50}). Mathematical and statistical treatments of the serum dose–syncytia inhibition response curves were performed after probit transformation of the response as described by Finney [22, 23]; details on the graphical method using logarithm-probability paper were given by Litchfield and Wilcoxon [24].

RESULTS

Respective roles of CC 81 and FLK-BLV cells in syncytia formation

In preliminary trials, mixtures of FLK-BLV and CC 81 cells were cocultivated so as to

*Table 1. Summarized procedure for the performance of early polykaryocytosis inhibition (EPI) assay of bovine sera**

Complete procedure (I)	Simplified procedure (II)
(1) i. 0.1 ml serial log 2 dilutions in culture medium† of the sera to be assayed are distributed following lines.	(1) i. 0.1 ml serial log 2 dilutions in culture medium† of a titrated reference serum and of the sera to be assayed are distributed following lines.
ii. at regular line intervals, one line is filled with PBS‡ or culture medium only (control non serum-treated mixed cultures).	
(2) 0.1 ml complete culture medium§ containing a number of FLK-BLV cells determined according to equation: $c = s^{k-1}$ (in which c is the number of FLK-BLV required to obtain s syncytia) is added per well.	(2) ii. as in (I)
(3) cultures are incubated for 1 hr (37°C)	(3) as in (I)
(4) 0.1 ml complete culture medium containing 2.0×10^4 CC 81 cells is added per well and the plate sealed.	(4) as in (I)
(5) After 24 hr total incubation the cultures are rinsed 3 times with PBS and stained with Giemsa.	(5) as in (I)
(6) The amount of syncytia per well is recorded and the percentage of syncytia inhibited per serum dilution is calculated.	(6) The SID_{50} of the reference serum and of the sera assayed are determined under low microscope magnification by gross comparison of the wells containing serum dilution and those with cells only.
(7) The percentages of inhibition versus serum dilution factors are plotted on a graph paper with probit versus log scale.	(7) The normalized titer $SID_{50}N$ is calculated for each serum with formula $SID_{50}N¶(A) = SID_{50}(A) \times \frac{SID_{50}N(R)}{SID_{50}(R)}$ where (A) denotes the serum assayed and (R) the reference serum.(8)
(8) The SID_{50}‖ is either calculated by profit analysis or estimated graphically.	
(9) The normalized titer ($SID_{50}N$¶) is calculated with formulae $SID_{50}N = SID_{50} \times \left(\frac{S}{s}\right)^K$ in which s refers to the total amount of syncytia per well in control cultures, S to the normalized number of syncytia per well and K a factor to be determined for once.	

*The assay is performed using Falcon Microtest II tissue culture plates.
†Culture medium: RPMI 1629.
‡PBS: phosphate buffered saline.
§Complete culture medium: RPMI 1629 supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum, 500 units/ml penicillin, 500 μg/ml streptomycin and 100 μg/ml fungizone.
‖SID_{50}: reciprocal of the serum dilution which gives a 50% reduction of the number of syncytia as compared to control cultures.
¶$SID_{50}N$: normalized SID_{50} as if S syncytia would have been obtained in control cultures.

obtain a confluent or nearly confluent monolayer after 24 hr incubation, a condition in which syncytial cells are easily visualized.

Concentrations between 0.5×10^4 and 2.0×10^4 CC 81 cells permitted an optimal visualization and numeration of syncytia. The trials were repeated varying the concentration of FLK-BLV cells. As shown in Table 2, the number of syncytia per well (s) increases with the concentration of both CC 81 and FLK-BLV cells. The average size of the syncytia was not constant. Indeed we noticed that they were small or very small when scarce, the number of nuclei increasing with the number of syncytia. The data also indicate that the ratio $100s$: number of FLK-BLV cells per well increases, but not proportionally with CC 81 cell concentration and decreases with FLK-BLV cell concentration. This kind of relationship suggests that if a syncytium originates from "contact" between the two kinds of cells, several CC 81 cells are required for its formation, whereas several FLK-BLV cells could participate in the induction of the same syncytium. This could also explain why the number and size of the syncytia increase simultaneously. The role of FLK-BLV cells as actual inducers of syncytia is also supported by experiments in which less than 100 ovine cells were seeded with numerous CC 81 cells or the proportions reversed. Indeed, whatever the amount of FLK-BLV cells, syncytia were observed only when the cat cells were in a sufficient number.

In the following experiments the concentration of 2.0×10^4 CC 81 cells per well was used because the number and size of syncytial cells obtained in cocultures even with a wide range of FLK-BLV cells concentration, was the most appropriate for an accurate numeration.

Correlation between FLK-BLV cell concentration and syncytia formation

As FLK-BLV cells appeared to be the actual syncytia inducers, it was important, for an ultimate use in different types of assays, to investigate the relationship between the number of syncytia appearing in cocultures of CC 81 and FLK-BLV cells and the concentration of the latter. Cocultures were seeded with 2.0×10^4 CC 81 cells per microtest well and a range of 15 FLK-BLV cell concentrations extending from 2.5×10^2 to 2.1×10^4 cells per well. Between 4 and 6 wells were prepared per concentration of FLK-BLV. The result was not a direct relationship between the number of syncytia per well (s) and the concentration of FLK-BLV cell (c), but the logarithm of s was found to be proportional to the logarithm of c (Fig. 1). The equation of

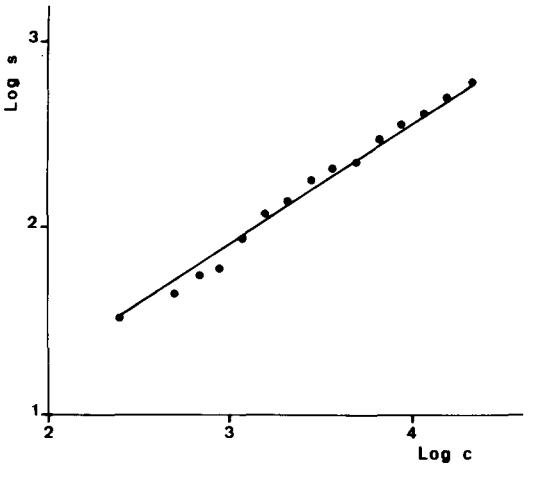

Fig. 1. Effect of the concentration of FLK-BLV cells on early polykaryocytosis in mixed cultures with CC 81 cells.

The experiment was performed in Falcon Microtest II tissue culture plates with 2.0×10^4 *CC 81 cells per well;* s *is the number of syncytia and* c *the amount of FLK-BLV cells per well; experimental data* (●)*; regression line, log* s = 0.6346 *log* c (—).

*Table 2. Influence of the concentration of CC 81 cells on early polykaryocytosis**

	CC 81‡		
FLK-BLV†	0.5×10^4	1.0×10^4	2.0×10^4
0.1×10^4	49§ (4.92)‖	99 (9.87)	116 (11.62)
0.2×10^4	144 (7.25)	271 (13.55)	232 (11.62)
0.5×10^4	166 (3.32)	331 (6.62)	436 (8.72)
1.0×10^4	225 (2.25)	337 (3.37)	515 (5.15)

*Assay performed in Microtest II tissue culture plates.
†Concentration of FLK-BLV cells per well.
‡Concentration of CC 81 cells per well.
§Number of syncytia per well.
‖Ratio $\frac{\text{number of syncytia per well}}{\text{number of FLK-BLV cells per well}} \times 100$.

the representative curve can be theoretically expressed as:

$$\log s = k \log c + b. \quad (1)$$

For two reasons b can be estimated to be nil or close to nil. First, the ratio $\log s : \log c$ was found approximately constant for the 15 concentrations and comprised between 0.604 and 0.653 and on the other hand, syncytia were not encountered in control CC 81 cultures. Therefore equation (1) becomes

$$\log s = k \log c \quad \text{or} \quad (2)$$

$$s = c^k. \quad (3)$$

Experimental data permit to estimate $k = 0.6346 \pm 0.0146$.

This relationship indicates that one FLK-BLV cell is sufficient to induce one syncytium and that all FLK-BLV cells are equivalent in their syncytia inducing activity. With increasing concentrations, more FLK-BLV cells seem to cooperate to the formation of each syncytium, a phenomenon which is associated with an increase of the nuclei content per syncytial cell. An important application of equation (3) is the theoretical evaluation of the concentration of FLK-BLV cells required to obtain a desired number of syncytia per well:

$$c = s^{k^{-1}} \quad (4)$$

In most of our experiments we used the combination 5.0×10^3 FLK-BLV cells with 2.0×10^4 CC 81 cells per well. In these conditions, syncytia are of a sufficient size (generally comprised between 10 and 100 nuclei per cell) and number and adequately dispersed in the monolayer for an accurate numeration.

Even when standardized conditions were used in assaying FLK-BLV cells, limited fluctuations of the amount of syncytia induced were often observed from one experiment to another. These fluctuations could be ascribed to differences in the state of FLK-BLV cells at the time of removal from the maintenance cultures; therefore the influence of cell age after subculturing was investigated. Parallel FLK-BLV cell cultures were initiated at different densities in such a way that any part of the cell growth curve could be obtained. At daily intervals and for each concentration, a culture was removed, the cells counted and kept frozen until assayed in the same experiment for their ability to induce syncytia on CC 81 cells. As shown in Table 3, apart from degenerating aged cultured (96 hr) in which cells may have a reduced syncytia inducing potency, the variation factor of the amount of syncytia per FLK-BLV cell does not exceed 1.5. This result further indicates that in their usual conditions of maintenance FLK-BLV cells retain a constant syncytia inducing ability.

Inhibition of syncytia formation with bovine sera

Sera from cattle with persistent lymphocytosis (LP) or with the adult form of lymphosarcoma (LSA) were assayed for their eventual ability to inhibit syncytia formation in cocultivated FLK-BLV and CC 81 cells. The results obtained with sera 461 (normal), 1121 (LP) and 11 (LSA) are representative. Whereas, the sera from clinically and hematologically normal cows from leukemia-free herds do not interfere with syncytia formation at any dilution, the sera from animals with LP or LSA inhibit syncytia formation with different potencies, and their effect is dependent on the concentration of the serum added. When the percentage of syncytia in-

*Table 3. Influence of the age of FLK-BLV cells in maintenance cultures on early polykaryocytosis upon cocultivation with CC81 cells**

Culture No.	Number of cells seeded : 10^6	Time of incubation (hr)			
		24	48	72	96
1	0.20	0.13†(835)‡	0.45 (768)	0.77 (944)	1.57 (613)
2	0.40	0.36 (839)	1.14 (763)	1.42 (969)	3.64 (770)
3	0.80	0.87 (973)	2.09 (807)	3.88 (872)	3.42 (381)
4	1.60	2.01 (699)	3.26 (724)	5.79 (805)	3.11 (490)

*FLK-BLV cells were seeded at various densities in Falcon tissue culture flasks (25 cm) and tested at regular time intervals for their syncytia inducing activity.
†Number of FLK-BLV cells per culture after the indicated time of incubation.
‡Number of syncytia per 5×10^3 FLK-BLV after the indicated time of incubation.

hibition is plotted against the logarithm of the serum concentration (Fig. 2) one can observe that the slopes of the linear part of the representative curves are comparable. This suggests that syncytia inhibition represents the same type of reaction for different sera. In the same type of assay, anti-BSV serum 449 does not interfere with syncytia formation.

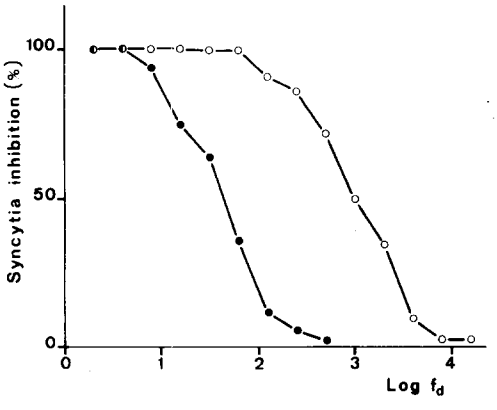

Fig. 2. Typical dilution assays of bovine sera using early polykaryocytosis inhibition. Plot of the percentage of syncytia inhibited versus the logarithm of the dilution factor (f_d). Serum 1121 *is from a cow with persistent lymphocytosis* (●) *and serum* 11 *from a case of bovine adult lymphosarcoma* (○).

These results support the hypothesis that FLK-BLV cells express on their surface or that they release substances responsible for syncytia induction recognized by antibodies contained in the sera of BLV infected animals. As syncytia are also induced by BLV infected bovine lymphocytes but not by normal lymphocytes cocultivated with CC 81 cells (unpublished results) it is logical to assume that syncytia inhibiting substances in the sera of diseased animals might be antibodies associated with BLV.

Absorption of bovine serum with BLV antigens

Bovine serum 1121 was preincubated with BLV antigens prior to assaying its syncytia inhibiting activity. Table 4 shows that preincubation of the positive serum with ether-treated BLV soluble antigens i.e. most likely internal antigens, resulted in a significant reduction of the titer; a similar result was obtained by use of a glutaraldehyde-treated BLV preparation, in which only viral envelope antigens are exposed to the serum action. The titer of the same serum was not modified when the preincubation was performed with a mouse leukemia virus (RadLV-D).

These data suggest that the inhibitory effect of a positive serum on syncytia formation is at least partially due to antibodies directed against some internal antigen(s) and BLV surface antigen(s), and conversely, that syncytia induction by FLK-BLV cells is mediated, at least in part, by these antigens.

Absorption of bovine serum with BLV infected cells or their growth medium

In an attempt to localize the BLV antigens responsible for syncytia formation, absorption of a bovine positive serum (1121) with BLV infected cells or their culture fluids was carried out prior to testing its inhibitory activity on syncytia induction. FLK-BLV cells, lymphocytes from cows with LP (333) or LSA (LB 44), both BLV infected as well as non BLV producing lymphocytes from cow (H) were used in these experiments. The lymphocytes were either not cultured, so no complete viral particles were released, or cultured for 48 hr, a condition for virus release by BLV infected lymphocytes.

*Table 4. Effect of absorption of a bovine serum with preparations of BLV antigens on its early polykaryocytosis inhibitory activity**

Treatment: preincubation with	Titer ($SID_{50}N$)†
—	218
(1) BLV ether-treated soluble fraction‡	69
(2) Glutaraldehyde treated BLV preparation§	50
(3) Glutaraldehyde treated RadLV-D preparation‖	203
(4) Mixture of (1)+(2)¶	53

*Serum 1121 is from a cow with persistent lymphocytosis.
†Normalized titer: reciprocal of the dilution giving a 50% reduction of the number of syncytia when cocultivation of CC 81 and FLK-BLV cells alone would result in the formation of 300 syncytial cells.
‡A vol of 50 ml FLK-BLV culture fluid was used.
§A vol of 100 ml FLK-BLV culture fluid was used.
‖A vol of 100 ml 13-3-C culture fluid was used.
¶Absorption performed with half the volume used in (1) and half the volume of (2).

The titers of the serum after the different absorption experiments are shown in Table 5. Uncultured lymphocytes have no significant effect on the serum titer whereas cultured LP or LSA lymphocytes or FLK-BLV cells reduce significantly the inhibitory activity of the serum on syncytia formation. Cultured lymphocytes which do not produce BLV have no effect. The culture fluid of BLV infected lymphocytes significantly reduces the titer of the absorbed serum. In contrast, the culture fluid of normal lymphocytes has no effect.

*Table 5. Effect of absorption of a bovine serum with BLV infected cells or their culture fluid on its early polykaryocytosis inhibitory activity**

Origin of cells or culture fluid used for serum absorption	Titer ($SID_{50}N$)†		
	Time of culture prior to absorption		
	0 (hr)	48 (hr)	Culture fluid‡
—	218	—	—
Lymphocytes H§ (20×10^6)*	261	235	194
Lymphocytes LB44‖ (40×10^6)	238	116	68
Lymphocytes 333¶ (40×10^6)	204	88	49
FLK-BLV (7×10^6)	—	126	—

*Serum 1121 was from a cow with persistent lymphocytosis.

†Normalized titer: reciprocal of the dilution giving a 50% reduction of the number of syncytia when cocultivation of CC 81 and FLK-BLV cells alone would result in the formation of 300 syncytial cells.

‡Bovine lymphocytes or FLK-BLV were incubated for 48 hr with 25 ml culture medium, one fifth (5 ml) of this volume was used in absorption experiments.

§Lymphocytes H are from a clinically and hematologically normal cow.

‖Lymphocytes LB44 are from an adult cow with lymphosarcoma.

¶Lymphocytes 333 are from a cow with persistent lymphocytosis.

**Figures between parentheses indicate the number of cells used in these experiments.

Our results suggest that the syncytia inducing activity of BLV infected cells is mediated in part by BLV antigens displayed at, or closely associated with, the cell membrane and in part by BLV or BLV antigens released by these cells. Quantitative data also support the possibility that in cocultures of BLV infected cells and CC 81 cells, BLV or BLV released antigens play a more pronounced role in syncytia induction than the cells themselves.

Probit transformation of the percentage of syncytia inhibition by bovine sera

More than 100 sera were assayed for their syncytia inhibiting activity. FLK-BLV cells at a concentration of 5.0×10^3 per well were preincubated with serial dilutions of log 2 intervals of the sera and cocultivated with 2.0×10^4 CC 81 cells per well.

No linear relationship was found between the number or the percentage of syncytia inhibited and the dilution. After logarithmic transformation of the dilution factor, the representative curve was partialy linear, and could permit an approximate estimation of the SID_{50} (Fig. 2). However, in these conditions only part of the information contained in the dilution assay could be taken into account. The examination of such a dose–effect relationship indicates that the rate of increase in response per logarithm of unit of dose (or of the dilution factor) is very low in the regions of 0 and 100% response and much higher in the intermediate regions. These characteristics are well exemplified in the representative serum assay curves which are of the sigmoid type.

Although the dosage–response in our serum assays is not strictly quantal, a probit transformation of the response can be applied for a better evaluation of the median effective dose according to Finney [22, 23]. Indeed, the use of probits instead of the real percentages, and of weighting coefficients for each observation result in the transformation of the normal sigmoid curve into an almost straight line and allows a more accurate determination of the SID_{50}. In these conditions a regression curve fitting all dose–response data for any serum can be drawn and the SID_{50} can be determined either graphically or by computer calculation.

The estimate of the logarithm mean effective dose or mean effective dilution factor (log SID_{50}) is the value of the intercept of the regression curve with $y = 5.0$ and the SID_{50} its antilogarithm.

The accuracy of the SID_{50} can be estimated using the variance (V) of log SID_{50} and the determination of a value of χ^2 to test for homogeneity of the data. The fiducial limits of log SID_{50} with 95% confidence are

$$\log SID_{50} \pm 1.96V. \quad (5)$$

Figure 3 shows such a probit transformation of the effect of bovine serum 647 on syncytia inhibition as a function of the logarithm of the

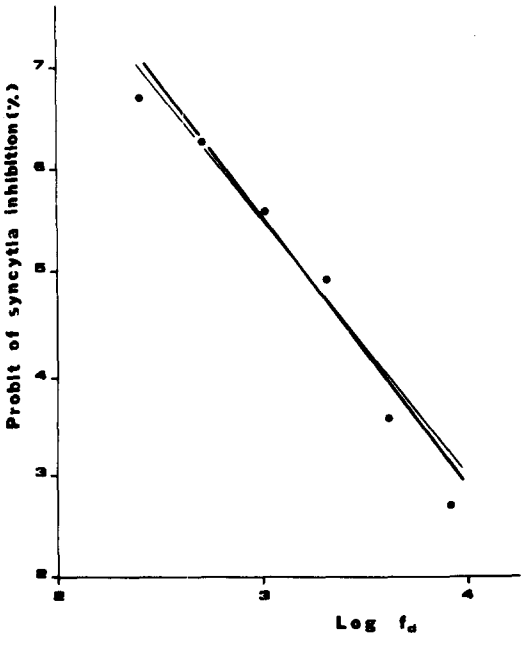

Fig. 3. Probit transformation of the percentages of syncytia inhibited by the serum of a cow with persistent lymphocytosis (647) after log 2 serial dilutions (f_d refers to the dilution factor; experimental data (●), empirical probit regression line (—), probit regression line (——).

serum dilution factor used. The regression was calculated and found linear according to equation

$$y = -2.615x + 13.346$$

and for $y=5.0$, x or log $SID_{50}=3.1916$, and $SID_{50}=1554$; the calculated linear regression is compatible with the experimental data ($P=0.02$) and the dilution factor corresponding to 50% inhibition is comprised between 822 and 2940 ($P=0.05$).

The fiducial limits found in this example are in the order of magnitude generally observed and the limits of the SID_{50} are roughly $\pm 50\%$ of its value (with 95% confidence). Therefore, we found convenient in most of the assays to replace the above methodology by a graphical method using "log-probit" graph paper on which the percentage of the probits is plotted against logarithm. In this way, the SID_{50} of serum 647 was evaluated as 1650 which is close to the calculated estimation and well inside of the fiducial limits. Figure 4 shows the graphical representation obtained with some sera from cattle with LSA (11,545), LSJ (5), LP (1121 and 647) and of a contact animal (640); it also shows that the slopes of the different representative assay curves are almost identical. The consistency of this observation favors that the syncytia inhibition reaction is of the same type and that the sera differ only by their relative syncytia inhibiting potencies.

Normalization of the expression of serum titers

Moderate fluctuations of the number of syncytia obtained with a fixed amount of FLK-BLV cells were frequently observed and probably due to inherent experimental errors even when standardized conditions of the assay were used. These variations were found to influence noticeably the results obtained in serum assays in such a way that the titer was diminished with increasing amounts of syn-

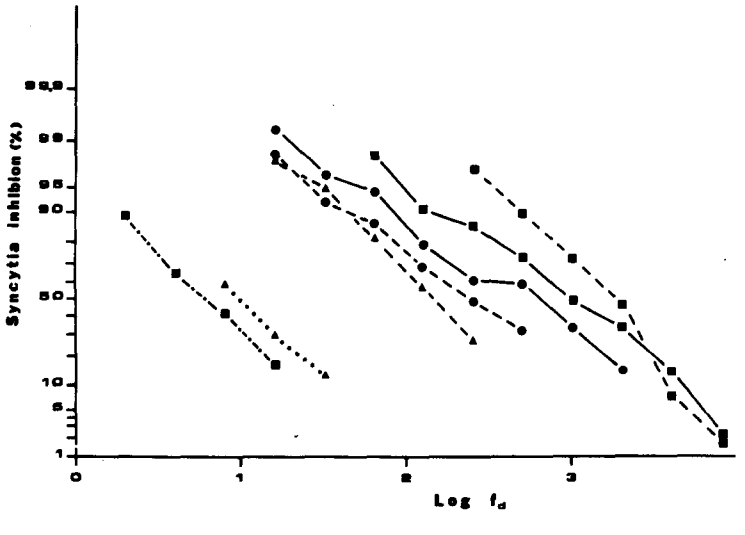

Fig. 4. Representation of the percentage of syncytia inhibition versus the logarithm of the serum dilution factor (f_d) as obtained by use of log-probit graph paper. Serum 640 is from a normal contact animal (-·-■-·-), 5 from a case of juvenile lymphosarcoma (···▲···), 647 (--●-- before centrifugation; --■-- after centrifugation) and 1121 (--▲--) are from animals with persistent lymphocytosis (—▲—), 545 (—●—) and 11 (—■—) are from cases of adult lymphosarcoma.

cytia in parallel non treated-control cultures and conversely. Thus, in order to compare the syncytia inhibiting activities of different sera, i.e. their potency, it was necessary to further investigate the influence of the number of syncytia on the estimation of the serum titer.

Serum 1121 was assayed in coculture combinations containing 2.0×10^4 CC 81 cells and 14 serial (log 1.33) FLK-BLV cell densities extending from 0.50×10^3 and 21.07×10^3 cells per well. After graphical measurement of the SID_{50} of serum 1121 in the different types of mixed cultures, a direct relationship between the logarithms of the titer (SID_{50}) and the amount of syncytia (s) could be ascribed in the limits of the FLK-BLV cell concentrations used (Fig. 5) according to equation:

$$\log SID_{50} = K \log s + b. \quad (6)$$

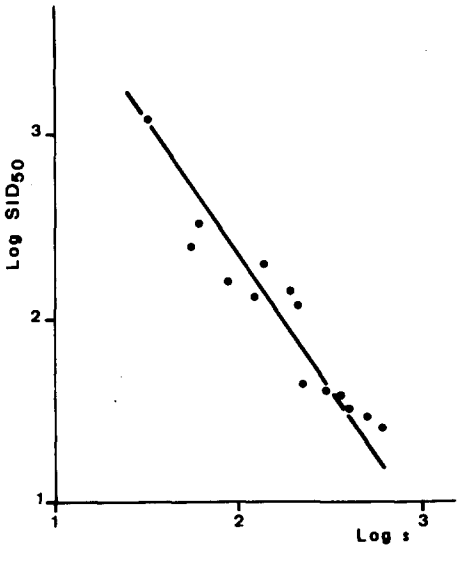

Fig. 5. Plot of the titer (SID_{50}) of serum 1121 (a case of persistent lymphocytosis) versus the logarithm of the number of syncytia in non serum-treated control cultures.

The calculus with experimental data indicates that a curve with equation:

$$\log SID_{50} = -1.2161 \log s + 4.7147$$

can be adjusted with 99% confidence; K must be comprised between -1.1052 and -1.2548 if 95% confidence limits are chosen.

In equation (6), b is dependent upon the inherent syncytia inhibiting potency of the particular serum tested and is not related to the number of syncytia obtained in the assay. Since the slopes (k) of the titration curves (equation 2) obtained with different sera were found almost constant, suggesting the same type of reaction, they also imply that the only differences between sera are their relative concentration in "syncytia inhibiting factor(s)" and therefore that the slope (K) of the representative curves corresponding to equation (6) remains constant whatever the serum assayed. In these conditions, the relation between the titer of a serum and the number of syncytia allows a normalization of the expression of the titer; with $SID_{50}N$ for the normalized titer of the serum, and S for the reference amount of syncytia, the equation (6) becomes:

$$\log SID_{50}N = K \log S + b \quad (7)$$

and from equations 6 and 7

$$SID_{50}N = SID_{50} \times (S/s)K. \quad (8)$$

This relation was applied to normalize the serum titers in different assays using $S = 300$, as such a quantity of syncytia is easily counted and allows a good estimation of the titers of sera with a low inhibiting activity.

Table 6 shows the consistency of $SID_{50}N$ calculated for several values of s with the serum (144$\hat{1}$) of a cow with persistent lymphocytosis.

Simplified early polykaryocytosis inhibition test

When a great accuracy of the serum titer was not required, like it is most often the case in routine serum titrations, the complete assay procedure was found quite unnecessary. Indeed, 100% and no inhibition can be easily seen without any count and the SID_{50} can be visualized fairly correctly even by a non trained experimentator.

As shown in Table 6, the SID_{50} determined by direct observation agreed well with those obtained by probit analysis and fell well inside of the 95% confidence limits. As the numbers of syncytia are not determined in this simplified assay, the titer of a serum must be estimated by comparison to a known reference serum previously titrated using the complete assay method. In a single experiment, if (R) and (A) denote the reference and the serum assayed, the following relation can be used:

$$SID_{50}N(A) = SID_{50}(A) \times \frac{SID_{50}N(R)}{SID_{50}(R)}. \quad (9)$$

Table 6 also shows that the use of the simplified EPI test gives $SID_{50}N$ estimates which are close to those obtained with the complete procedure.

Table 6. Normalization of the expression of serum titers

Serum No.	Experiment No.	S*	Complete EPI assay		Simplified EPI assay	
			SID_{50}†	$SID_{50}N$	SID_{50}‡	$SID_{50}N$
1441	1	509	82	156	128	— (243)§
	2	595	82	189	64	— (147)
	3	615	86	206	64	— (153)
	4	626	64	157	64	— (157)
	5	894	44	166	32	— (121)
	6	909	34	131	32	— (123)
	7	1200	33	178	32	— (173)
	mean		61±24	169±25	59±34	—(160±41)
136	1	509	12	23	16	21‖(30)§
506	1	509	15	29	16	21 (30)
542	1	509	10	19	8	11 (15)
645	1	509	21	40	32	42 (61)
549	1	509	24	46	32	42 (61)
650	1	509	52	99	64	84 (122)
1118	1	509	122	232	128	169 (243)

*Average number of syncytia per well in non serum treated control cultures for each experiment.

†Titer determined by the graphical method before normalization.

‡Titer determined by gross eye observation before normalization.

§Between parentheses: titer estimated by reference to the average number of syncytia per well in non serum treated control cultures.

‖Titer determined by comparison to a reference serum (1441) previously titrated with the complete EPI assay procedure ($SID_{50}N = 169$).

Application of the EPI test to a preliminary survey of BLV infection in cattle

In a cooperative work, 11 reference bovine sera, kindly supplied to us by Dr. C. Olson, were blind assayed using both the EPI and the complement fixation technique. Table 7 illustrates the confrontation of our results with those obtained in 13 other laboratories with techniques routinely used in serological surveys aimed at the detection of BLV infected cattle (immunodiffusion, immunofluorescence and complement fixation) and totalizing 23 separate assays. Whereas we detected complement fixing antibodies only in the sera recorded as positive by other laboratories, early polykaryocytosis inhibition was observed with all. Indeed 2 sera were found completely negative in all other tests and one had only a slight complement fixing activity as detected by 3 laboratories including ours. It is noticeable that these sera which had low but significant $SID_{50}N$ originated from one calf with lymphosarcoma and two contact control animals previously non suspected of BLV infection. This observation supports the hypothesis whereby the EPI test detects antibodies that may not be revealed by the aforementioned methods.

The EPI and CF techniques were used in parallel in a preliminary survey including 105 bovine sera (Table 8). These sera were obtained from hematologically and clinically normal animals from leukemia-free herds (N), normal contact animals (NC), animals with persistent lymphocytosis (LP), with the adult from of lymphosarcoma (LSA), juvenile lymphosarcoma (LSJ) and cutaneous lymphosarcoma (LSC).

The results obtained with the EPI test are the following: among the animals which could be considered as suspect of BLV infection, the 6 with LSA were found positive with generally high titers (average 977); all those with LP were also positive with somewhat lower titers (average 104); the 4 LSJ, the LSC and all but one NC were positive with low titers (respective averages 16, 16 and 28); none of the N had a significant titer.

A similar pattern was obtained with the complement fixation technique, but the percentages of positive sera were smaller in all animal categories. Better agreement was found for clinically leukemic adult and hematologically suspect animals. The percentage was much smaller in animals with a normal hematologic picture but which could have been infected with BLV (NC). None of the LSJ or LSC were detected.

As it could be expected, a highly significant correlation was found between titers obtained in EPI and CF tests. Although it is possible that the two assays do not reveal quanti-

*Table 7. Comparison of tests on bovine reference sera performed in 14 laboratories**

Serum No.	Results obtained by outside laboratories										GELB results	
	ID (gs)† 9**	ID (es-gp)‡ 6	IF§ 4				CF‖ 4				CF	EPI¶
1	+††	+	64‡‡	128	160	+	32	128	32	+	32	34
2	−	+(5)/−(1)	8	32	−	+	0	16	16	−	16	47
3	+(8)/−(1)	+	64	256	160	+	64	128	32	+	128	107
4	+	+	32	256	320	+	16	32	0	+	32	192
5	−	−	−	−	−	−	2	2	0	−	2	20
6	+	+(5)/+?(1)	128	512	1280	+	64	256	64	+	256	256
7	−	+?(1)/−(5)	−	−	−	−	0	0	0	−	0	8
8	+	+	128	512	320	+	16	64	32	+	32	86
9	+(1)/±(4)/−(4)	+(5)/−(1)	8	32	−	+	4	64	16	+	16	14
10	−	−	−	−	−	−	0	0	0	−	0	0
11	+	+	64	512	1280	+	64	256	32	+	32	1530

*Modified and extended with permission of Dr. C. Olson (Olson and Baumgartener, 1975).
†Gel diffusion test using an ether-resistant antigen of bovine leukemia virus (Olson C. *et al.*, 1973).
‡Gel diffusion test using an ether-sensitive antigen associated with bovine leukemia virus infection (Onuma *et al.*, 1975).
§Immunofluorescence test (Ferrer, 1972).
‖Complement fixation test (Miller and van der Maaten, 1974).
¶Early polykaryocytosis inhibition test.
**Number of participating laboratories.
††"+" or "−" only indicate a complete agreement of the participating laboratories; in case of discrepancies, the number of laboratories with the same result is indicated between parentheses.
‡‡Each column reproduces the titers communicated by one laboratory.

Table 8. Incidence of BLV natural infection among cattle as evaluated by early polykaryocytosis inhibition (EPI) and complement fixation (CF) tests

Animal categories			Titer* distribution								No positive sera/no sera tested†	% of positive sera
			2	2–20	20–40	40–100	100–200	200–300	300–1000	>1000		
Non-suspect of BLV infection‡	N‖	EPI	31	—	—	—	—	—	—	—	0/31	0
		CF	31	—	—	—	—	—	—	—	0/31	0
Suspect of BLV infection§	NC‖	EPI	1	27	1	2	1	1	—	—	32/33	97
		CF	27	5	1	—	—	—	—	—	6/33	18
	LP‖	EPI	—	5	6	9	5	3	2	—	30/30	100
		CF	2	9	8	5	5	1	—	—	28/30	93
	LSA‖	EPI	—	—	—	1	—	1	2	2	6/6	(100)¶
		CF	1	1	1	—	—	1	1	1	5/6	(83)¶
	LSJ‖	EPI	—	3	1	—	—	—	—	—	4/4	(100)¶
		CF	4	—	—	—	—	—	—	—	0/4	(0)¶
	LSC‖	EPI	—	1	—	—	—	—	—	—	1/1	(100)
		CF	1	—	—	—	—	—	—	—	0/1	(0)

*For EPI tests, all titers were normalized in $SID_{50}N$ units with S = 300.
For FC tests, the titers were expressed as the reciprocal of the dilution preventing hemolysis of 50% of the red cells.
†All sera were coded and assayed in a blind manner.
‡Non suspect of BLV infection on epidemiological basis, the corresponding sera are from animals of leukemia-free herds.
§Suspect of BLV infection on epidemiological, hematological and/or clinical basis.
‖N: normal animals; NC: normal contact animals; LP: lymphocytotic animals; LSA: adult animals with lymphosarcoma; LSJ: animals with the juvenil form of lymphosarcoma.
¶Limited accuracy of the percentages in parentheses, due to the size of the sample.

tatively and/or qualitatively the same antibodies, both detect BLV infection, and the main difference for use in a survey lies in their threshold of sensitivity. Indeed, in most cases, when the $SID_{50}N$ of a serum is nil, the titer is also nil by CF and, on the average, complement fixing antibodies are only detected when a titer higher than 60 is obtained with the EPI test.

DISCUSSION

Formation, morphology and frequency of syncytia appearing in cocultures of the BLV infected ovine cell line, FLK-BLV, and the murine sarcoma virus infected cat cell line, CC 81, are noticeably different from those described by several authors [1, 2, 9, 25, 26]. Indeed, in contrast to the previous observations of small or medium size syncytia, detectable only after a relatively long incubation period and in most studies in an unpredictable amount, our previously reported results [11] show that giant cells appear as soon as one hour after cocultivation of FLK-BLV and CC 81 cells. The maximum amount of syncytia is consistently observed after 24 hr incubation, thus allowing routine and reliable numerations. The size and the nuclei content of these giant cells are also larger, since cells containing more than 100 and up to 500 nuclei may be abundant in cocultures depending on the cell densities. The rapidity of appearance of the syncytial cells and the marked similarity between their nuclei and those of single CC 81 cells suggest that polykaryocytosis occurs by fusion of CC 81 cells rather than by repeated endomitosis. These aspects of "early" and extensive polykaryocytosis contrast with other observations where the phenomenon would be rather of the "late" type.

Among other hypotheses, the syncytia inducing factor(s) could be either BLV itself, BLV antigens, or (and) tumor specific cell surface antigens. Our data indicate that BLV itself can be responsible for syncytia induction. Indeed, the inhibitory effect on polykaryocytosis of spontaneous sera absorption with glutaraldehyde fixed highly purified BLV, prepared according to a technique known to preserve the integrity of the virus and to maintain the antigenicity of the viral envelope [17]. In addition, the implication of some internal component(s) of the virions is likely in view of the positive results obtained in similar absorption tests carried out with ether-resistant BLV antigens.

FLK-BLV cells, as well as BLV producing lymphocytes, absorb the syncytia inhibitory activity of anti-BLV sera. Thus, such cells probably display either on their surface or, in close association, antigens recognized by antibodies contained in the sera. The finding that the culture medium used for the growth of BLV producing cells has a higher absorbing potency than the cells themselves supports the possibility that BLV or BLV released antigens play the most important role in syncytia induction. In these conditions, BLV producing cells would act as "inducing centers" and the syncytia effect would be largely mediated through synthesis and release of BLV antigens.

The role of BLV in syncytia induction is also supported by the following: careful electron microscopic examinations of FLK-BLV cell cultures and of all lymphocyte cultures used in our study did not reveal the presence of viral particles of another type; there is a complete accordance between the presence of BLV-antibodies in the sera of cattle and the syncytia inhibiting activity; a specific anti-bovine syncytial virus antiserum [10] does not interfere with syncytia formation; and, as shown previously [3, 9, 25, 27] antigens of the bovine foamy-like or the bovine medi-like syncytial viruses do not react with BLV-antibodies and conversely. The quantitative data discussed below also rule out the possibility of a virus released by CC 81 cells to be inducer.

Onuma *et al.* [28] showed that sera with precipitin or complement fixing antibodies to BLV also contain a factor responsible for virus release inhibition. Although this factor is not yet identified, its implication in syncytia inhibition by spontaneous anti-BLV sera requires further studies.

The quantitative investigation of the interactions between FLK-BLV and CC 81 cells leading to syncytia formation shows that the frequency and size of syncytial cells are dependent upon the relative concentrations of the two kinds of cells, and that several CC 81 cells are required for the formation of each syncytium. When a fixed amount of CC 81 cells is used, the relationship between the number of syncytia and that of cocultivated FLK-BLV cells (formulae 3) indicates that: all FLK-BLV cells are equivalent in their syncytia inducing ability and only one is sufficient; with rising FLK-BLV cell concentrations, and in agreement with the observation of an increase of the nuclei content per syncytial cell, more and more FLK-BLV cells participate to the induction of each syn-

cytium. An important application of formulae (4) for the performance of different assays is the theoretical evaluation of the number of FLK-BLV cells required in order to obtain a determined number of syncytia.

As early polykaryocytosis is specifically associated with BLV expression or replication and is inhibited in a quantitative manner by sera from cattle known to contain antibodies to BLV, it seemed useful to develop a rapid and simple assay for the detection of BLV infection.

In preliminary serological tests based on early polykaryocytosis inhibition (EPI), FLK-BLV cells were preincubated with serial dilutions of the sera to be assayed and, to these mixtures, CC 81 cells were added; after 24 hr the percentage of syncytia inhibition was determined for each serum dilution. The unit chosen for the expression of the activity of bovine sera was the reciprocal of the dilution inhibiting 50% syncytia as compared to control cultures (SID_{50}). The reason for this is that the median effective dose can be estimated with more accuracy than other doses and in particular the 100% effective dose. A difficulty was immediately encountered due to the large fluctuations in the titers of the same sera when assayed in different experiments. The most probable causes for such a lack of reproducibility were: (1) variations in the amount of syncytia induced and (2) insufficient accuracy in the determination of SID_{50}.

A first improvement was obtained in the performance of the assay by the use of standardized conditions concerning the time of serum preincubation, the concentrations of FLK-BLV and CC 81 cells and the total length of the incubation period. The amount of syncytia recorded in this way per serum dilution and in control cultures became nearly constant.

The criterion of accuracy was difficult to approach in the absence of a linear or of a simple relationship between the number or the percentage of syncytia inhibited and the dilution of the serum. Indeed, in these conditions, only few dilutions would be taken into account for the calculation of the titer. The use of the logarithm of the dilution factor in place of real dilutions resulted in a sigmoidal response curve as often encountered in immunological assays. Such a curve can readily be approximated after probit transformation of the percentages by a weighted regression straight line. A statistical treatment of the sigmoid response curve, as described by Finney [22, 23], permitted the determination, with a good accuracy, of the median effective dose (or dilution) and fiducial limits on the basis of all the responses to the dilutions assayed. However, some fluctuations were still recorded from one experiment to another experiment. Indeed, even relatively small variations of the amount of syncytia induced in separate EPI tests were associated with noticeable inverse deviations of the titers of the same serum.

Therefore, the influence of the number of syncytia (s) on the titer (SID_{50}) was further studied. A direct relationship between the logarithms of the two parameters was found; this allowed the normalization of the expression of serum titers ($SID_{50}N$) by reference to a determined number of syncytia (S).

Depending on the accuracy required, a simplified procedure was found advantageous in most routine assays. The basic principles are similar to that described above, but the SID_{50} is directly eye estimated. The potency of the serum is appreciated by comparison with the SID_{50} of a reference serum concomitently assayed. The basic steps followed in the performance of the complete and of the simplified assay procedures are outlined in Table 1.

The incidence and potency of natural early polykaryocytosis inhibiting sera was investigated in a preliminary seroepidemiological survey of BLV infection carried out with 105 sera by use of the complete EPI test procedure. Although limited, our data indicated that BLV infection was encountered in all animal categories in which it was logical to expect it on hematological, pathological and/or epidemiological basis, i.e. leukemic (100 %), lymphocytotic (100 %) and normal contact animals (97 %). On the contrary, all sera from normal animals randomly selected in leukemiafree herds were negative. Among the categories of reactors, significant differences of the normalized titers were observed; those corresponding to the adult form of lymphosarcoma were on the average higher than those of lymphocytotic animals (977 and 104 respectively); the lowest titers were comparable in normal contact animals and in the few cases of juvenile or cutaneous lymphosarcoma tested (28, 16 and 16).

These seroepidemiological data were compared with those obtained in parallel experiments by complement fixation, a technique largely used in surveys. A highly significant correlation was found between the titers obtained by the two procedures; the main differ-

ence was the lower threshold of sensitivity of the EPI test. Apart from juvenile and cutaneous lymphosarcoma, reactors were found with both techniques in the same categories of animals; however, more positive sera were detected with the EPI test than with the complement fixation test, especially in normal contact animals. Statistically, the complement fixation technique would detect only those animals with a $SID_{50}N$ equal or higher than 60.

The high sensitivity of the EPI method for the detection of BLV associated antibodies was further indicated in a comparative study performed on 11 sera independently tested by 14 laboratories by use of complement fixation [8], immunodiffusion [7] and immunofluorescence [5, 6], techniques.

The results of our survey agree well with those reported by Cornefert-Jensen *et al.* [2] using the inhibition of late syncytia formation. All cattle with the adult form of lymphosarcoma were also detected, but not those with the calf type. The incidence of reactors among contact animals was much smaller than reported here; however, such a discrepancy could be ascribed to differences in the animals sampled.

The seroepidemiological data gathered with the EPI test are in accordance with those of tests for antibodies involving precipitins, complement fixation and immunofluorescence as well as those based on hematological criteria, They also fit with field epidemiological observations. However, it remains to be elucidated whether reactor animals have or have had infection with the virus. Our observations of positive reactions with the sera of calves with lymphosarcoma and of one case of cutaneous lymphosarcoma (two sporadic forms of the disease) supports the hypothesis that spread of bovine leukemia not only occurs on an horizontal mode but the etiological agent of the disease could also be vertically transmitted on a latent form and its expression activated in rare and still unknown circumstances. This view is further supported by the transmission experiments performed by Miller *et al.* [29] who succeeded in infecting experimental calves with BLV after inoculation with tumor material removed from calves with lymphosarcoma.

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Effect of Immunomodulators on Thymocyte Suppressive Activity in Mice*

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Abstract—Corynebacterium parvum (C. parvum), *Bacillus Calmette-Guerin (BCG) and Pyran copolymer (Pyran) are capable of significantly reducing thymocyte suppressive activity as assessed by the capacity of C3H thymus cells to inhibit the local graft vs host reaction (GVHR) mounted by C3H spleen cells in immunosuppressed H-2 histoincompatible hosts. This effect, which is dose related, is already evident* 2 days *after the injection of* C. parvum *or BCG and* 4 days *after administration of Pyran; it lasts* 1–3 weeks, *depending on the immunomodulator and is accompanied by a significant reduction of thymus cellularity. Evaluation of GVHR kinetics shows that thymus suppressive activity is inhibited by these agents at all phases of the reaction. No reduction of thymocyte suppressive activity is observed in adrenalectomized mice treated with* C. parvum *or Pyran.*

INTRODUCTION

THE ANTINEOPLASTIC efficacy of treatments with non specific immunomodulators has been repeatedly demonstrated in various experimental systems and in at least a number of clinical malignancies [1–5]. The mechanism(s) of action of these agents is complex and has not yet been entirely elucidated. Specifically, the effects of immunomodulators on suppressor cells have so far received only limited attention [6–8] although the activation or inhibition of lymphoid cells with suppressive activity has been reported to play an important role in neoplastic progression and in other pathological conditions [9–15]. The identification, therefore, of agents capable of modulating the activity of these cells could be of potential therapeutic interest.

On this basis and in an effort to acquire more information on the mode of action of this type of agents we investigated the effect of *C. parvum*, BCG and Pyran on murine thymocyte suppressive activity. Evidence will be presented that treatment with these agents can markedly reduce thymus suppressive activity, employing the inhibition of a local GVHR as suppressor cell assay.

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MATERIALS AND METHODS

Animals

C3H ($H\text{-}2^k$) and $(C57Bl/6 \times DBA/2)F_1$ (hereafter referred to as $B6D2F_1$ ($H\text{-}2^{bd}$)) female mice, 18–20 g of weight, 5–7 weeks old, obtained from Charles River, Calco, Italy, were used throughout.

Drugs

C. parvum (Lot CA 582 B) was obtained from Wellcome Research Laboratories, Beckenham, England. Fresh BCG was supplied by Pasteur Institute, Paris, France. Pyran (NSC 46015) was a gift of Dr. R. Geran, NCI, Bethesda, Md. Unless otherwise specified, the dose of *C. parvum* used was 1.4 mg/mouse given i.p. for two consecutive days, the dose of BCG was 1 mg/mouse i.v. and that of Pyran was 50 mg/kg i.p. These agents were resuspended or dissolved in sterile non pyrogenic saline. Control mice received equivalent amounts of sterile saline. Cyclophosphamide (Cy), kindly provided by Dr. H. J. Wood (NCI, Bethsda, Md.) was dissolved freshly in sterile saline before i.p. injection.

Assay of suppressor thymocytes

The concomitant injection of normal thymus cells has been shown to reduce

splenocyte-induced GVHR, an effect ascribed to the presence of a subset of suppressor cells in the thymus [16]. The popliteal lymphnode weight gain assay as described by Hardt and Claësson [17] was used as a measure of local GVHR. B6D2F$_1$ mice immunodepressed with 100 mg/kg i.p. of Cy 24 hr before, were injected into the right hind footpad with 5 $\times 10^6$ spleen cells from C3H mice in 50 μl Hanks' balanced salt solution (BSS), the controlateral footpad being injected with an equal number of splenocytes inactivated by exposure to 4% formol (30 min at room temperature). This number of splenocytes had previously been shown consistently to induce the highest GVHR in these conditions, higher inocula not being significantly more effective. Seven days later recipient mice were killed, both popliteal nodes were excised, carefully trimmed of accompanying tissue and immediately weighed to an accuracy of 0.01 mg. Severity of GVHR was expressed by the lymphnode index (L.I.), i.e. the ratio between the right and left node weights. In preliminary experiments it had been found that the L.I. of B6D2F$_1$ mice injected with 5×10^6 normal or inactivated syngeneic splenocytes was consistently in the 0.8–1.1 range.

When thymus suppressor cell activity was tested the organ was removed carefully excluding parathymic nodes adhering to the capsule, and 1.25×10^6 washed thymocytes from control or treated C3H mice were mixed with 5×10^6 syngeneic spleen cells and injected into the B6D2F$_1$ recipients in a total volume of 50 μl BSS. This number of normal thymus cells had previously been found to give optimal inhibition in these conditions.

Adrenalectomy

C3H mice were surgically adrenalectomized under nembutal anesthesia, kept in the dark and given sterile 0.9% NaCl solution *ad libitum* until used.

C. parvum or Pyran treatment was given 8 days after surgery and thymus cells were collected 2 or 4 days later respectively. The completeness of surgery was checked by gross inspection and animals with adrenal gland residues were discarded. Ectopic adrenal tissue has been reported to be rare in this strain of mice [18]. Shame-adrenalectomized controls were also prepared and kept under the same conditions.

Statistical analysis

Data are reported as the mean ±S.E. of at least 5 animals per group and statistical significance of the differences between L.I. values was assessed by the Duncan New Multiple Range Test [19].

RESULTS

As shown in Fig. 1 the administration of *C. parvum*, BCG or Pyran is followed by a reduction in thymocyte suppressive activity. In fact, when thymocytes from C3H mice treated with one of these agents were mixed with spleen cells, L.I. values similar to those of GVHR positive controls were obtained. This effect was first seen two days after the administration of *C. parvum* or BCG and four days after the administration of Pyran. The reduction of suppressive activity lasted up to day 14 after treatment with *C. parvum* and up to day 7 after treatment with BCG or Pyran. Similar results were obtained when *C. parvum* was given i.v. instead of i.p. and when increasing doses of treated thymocytes were

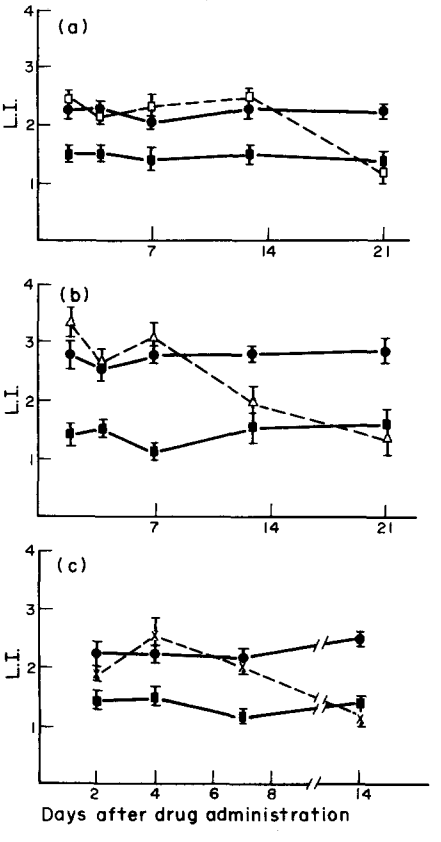

Fig. 1. Kinetics of the effect of C. parvum *(A), BCG (B) and Pyran (C) on thymocyte suppressive activity.* 5×10^6 *splenocytes were injected alone* (●——●) *or mixed with* 1.25×10^6 *thymocytes from normal mice* (■——■) *or from mice treated with* C. parvum (□----□) *BCG* (△- - -△) *or Pyran* (×----×). *GVHR was evaluated* 7 days *after cell transfer.*

mixed with spleen cells. In fact as shown in Fig. 2 by one representative experiment with

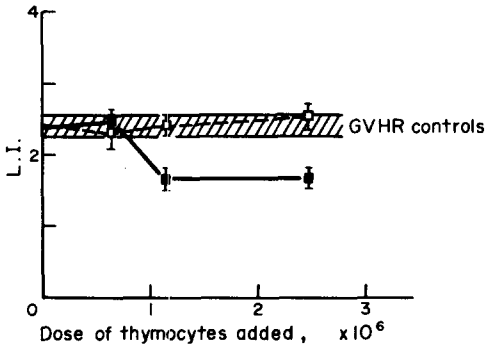

Fig. 2. Effect of different amounts of C. parvum *treated thymocytes on GVHR.* 5×10^6 *splenocytes were injected alone (GVHR controls, shaded area) or mixed with* 0.65, 1.25, 2.5 $\times 10^6$ *normal* (■———■) *or* C. parvum *treated* (□–––□) *thymocytes.*

C. Parvum, significant reduction of thymocyte suppressive activity was still seen when double amounts of *C. parvum* thymocytes were employed. Evaluation of thymus cellularity (Fig. 3) showed a significant decrease of thymus cell numbers in treated animals which roughly paralleled the reduction of thymocyte suppressive activity.

Localization of radiolabelled *C. parvum* in the thymus after parenteral administration in rodents has been described [20] and lymphoid tissue enlargement in animals given immunostimulants has been reported [21, 22]. Therefore in order to ascertain that the higher L.I. values observed employing thymocytes from *C. parvum* or BCG treated animals were not attributable to transfer with the cell suspensions of microorganisms or bacterial products not removed during the cell washings, the following experiments were performed. C3H thymocytes (1.25×10^6) obtained 2–7 days after *C. parvum* or BCG injection were mixed with C3H normal spleen cells and injected into Cy-pretreated C3H mice, the controlateral footpad being injected with mixtures of equal numbers of cells from normal mice. As controls a group of C3H mice was injected with normal thymocyte-spleen cells mixtures in both footpads. In these experimental conditions, involving no histocompatibility differences, no significant increases of L.I. were recorded 7 days after cell transfer comparing controls and mice injected with *C. parvum* or BCG thymocytes (Table 1). This was taken as an indication that thymus cell preparations did not contain *C parvum* or BCG microorganisms or their products in amounts

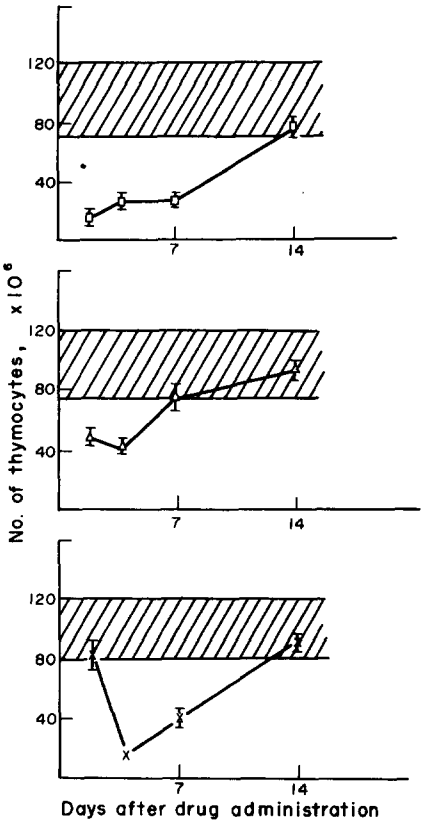

Fig. 3. Kinetics of the effect of C. parvum (□———□), *BCG* (△———△) *and Pyran* (×———×) *on thymocyte number in* C3H *mice. Shaded areas represent the numbers of thymocytes in untreated mice.*

capable of directly inducing significant lymph-nodal enlargement. Similar results were also obtained employing thymocytes from Pyran treated mice.

On the basis of these previous observations, in the next series of experiments thymocytes from treated animals were obtained 2 days after the administration of *C. parvum* or BCG and 4 days after Pyran treatment.

Within the dose range examined the effect of these immunostimulants on thymus suppressive capacity on a GVHR appeared to be dose related (Fig. 4). In fact the lowest dose of these agents resulting in a reduction of GVHR-suppressive capacity was 0.7 mg/mouse of *C. parvum* or 12.5–25 mg/kg of Pyran, although L.I. values superimposable on those of GVHR-positive controls were observed when doses of 1.4 mg/mouse of *C. parvum*, given for one or two days and 1 mg/mouse or 50 mg/kg of BCG or Pyran respectively were administered.

Considering that in the experiments described the L.I. were evaluated only at the peak of the GVHR, i.e. 7 days after cell transfer, in subsequent tests the entire GVHR kinetics was followed in mice injected either

Table 1. *Effect of* C. parvum *or BCG thymocytes on lymphnodal enlargement in C3H mice*

	Exp. Group	*C. parvum*	BCG	Lymphnode index Day 2	Lymphnode index Day 7
1	Splenocytes + Thymocytes	−	−	0.80±0.13	1.22±0.18
2	Splenocytes + Thymocytes	+	−	1.07±0.1	0.90±0.1
3	Splenocytes + Thymocytes	−	+	0.84±0.11	0.92±0.05

5×10^6 splenocytes were mixed with 1.25×10^6 thymocytes from normal, *C. parvum* or BCG treated mice and injected in Cy-immunosuppressed C3H mice.

Group 1 received mixtures of splenocytes and normal thymocytes in both footpads. Groups 2 and 3 received mixtures of splenocytes and normal thymocytes in one footpad and of splenocytes and *C. parvum*-thymocytes or BCG thymocytes in the controlateral footpad.

Thymocytes were obtained 2 or 7 days after *C. parvum* or BCG administration.

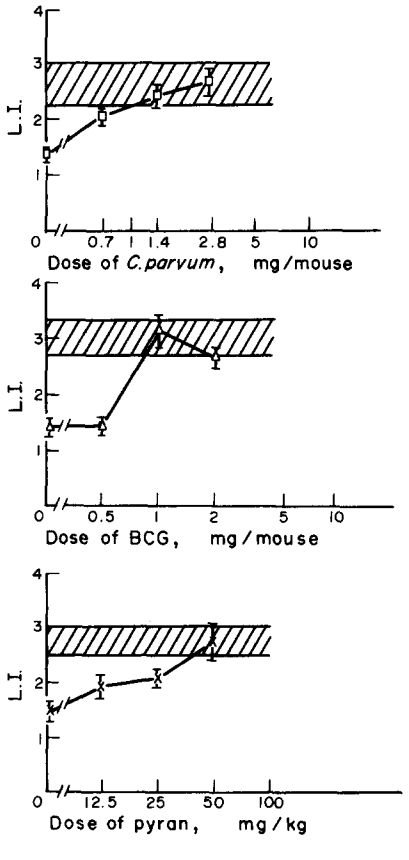

Fig. 4. *Effect of different doses of* C. parvum, *BCG or Pyran on thymocyte suppressive activity.* 2.8 mg/mouse *of* C. parvum *given in two* 1.4 mg *doses on* days −1 *and* 0. *Other treatments given on day* 0. *Thymocytes obtained on* day 2 *after* C. parvum *or BCG and* 4 days *after Pyran. Shaded areas represent GVHR controls.*

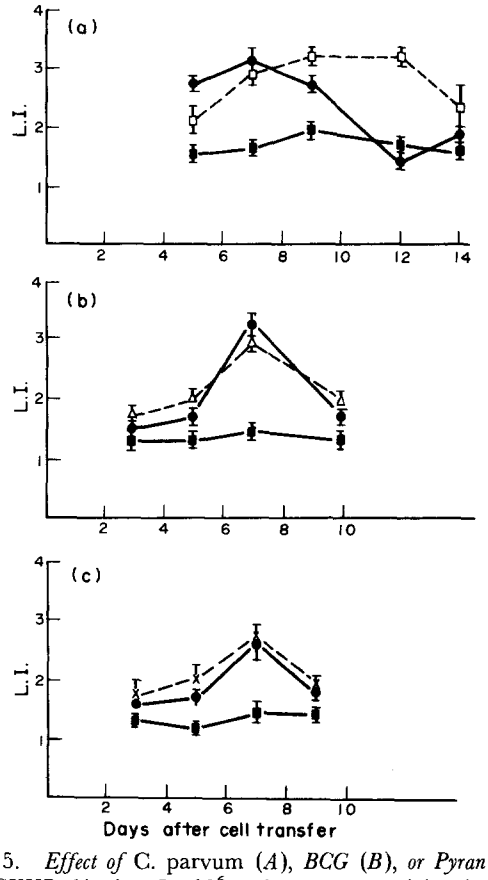

Fig. 5. *Effect of* C. parvum (*A*), *BCG* (*B*), *or Pyran* (*C*) *on GVHR kinetics.* 5×10^6 *splenocytes were injected alone* (●——●) *or mixed with* 1.25×10^6 *thymocytes from normal mice* (■——■) *or mice treated with* C. parvum (□----□) *BCG* (△----△) *or Pyran* (×----×). *Thymocytes obtained on* day 2 *after* C. parvum *or BCG and* 4 days *after Pyran.*

with spleen cells alone or with mixtures of splenocytes and thymocytes obtained from normal or treated animals. Figure 5 shows that when 1.25×10^6 normal thymocytes were transferred with 5×10^6 syngeneic splenocytes into the $B6D2F_1$ recipients a true depression of the GVHR ensued and not merely a delay in its peak. Conversely, when an equal num-

ber of thymus cells from drug-treated mice was injected, the GVHR kinetics observed was similar to that displayed by the GVHR positive controls except for a somewhat longer plateau observed when mixtures of splenocytes and *C. parvum* thymocytes were transferred. In fact, peak L.I. values were observed from day 7 up to day 12 and the first decrease in L.I. was detected only on day 14, i.e. 2–3 days later than in GVHR positive controls.

In view of the fact that thymus derived suppressor cells have been described as being steroid sensitive [23–25] it was of interest to obtain preliminary information on whether adrenal glands could be involved in the reported effect of these immunomodulators. Accordingly, the GVHR suppressive activity of thymocytes obtained 2 days after the injection of *C. parvum* (1.4 mg/mouse) or 4 days after the injection of Pyran (25 mg/kg) in adrenalectomized C3H mice was assessed measuring the L.I. 7 days after cell transfer. BCG-thymocytes were not employed because a relatively high mortality occurs in adrenalectomized mice treated with this agent. As shown in Table 2, no decrease in thymocyte suppressive activity could be observed in adrenalectomized mice treated with *C. parvum* or Pyran as compared to sham-adrenalectomized controls.

compatible hosts. This disappearance of suppressive capacity is dose-related and compounded by the marked reduction in thymus cellularity which follows treatments with these immunomodulators. Any discussion of the mechanism(s) through which these immunomodulators exert this effect on suppressor thymocytes would at the present time be mainly speculative. Since only a functional assay for suppressor cells was employed, it is impossible to state whether the reduction in thymus suppressive capacity in these experiments was due to inhibition or to destruction of these lymphoid elements and/or to their migration from the organ. The finding that suppressive activity is not reduced in adrenalectomized mice treated with *C. parvum* or Pyran suggests that adrenal glands may be involved. The lytic capacity of steroids for murine cortical thymocytes is well established and evidence has been provided that thymus derived suppressor cells or their precursors are steroid sensitive [23–25]. However, migration of suppressor thymocytes to the periphery cannot be excluded, as suggested by the work of Khalil *et al.* [21] whose findings favour thymocyte migration to T-dependent areas of the spleen in BCG treated mice. Also, the increased T suppressor cell activity found in the spleen of BCG treated mice [7] could be

Table 2. Effect of C. parvum *and Pyran on thymus suppressive activity in adrenalectomized mice*

Group	Adrenalectomy	*C. parvum*	Pyran	Lymphnode index
Spleen	–	–	–	2.4±0.2
Spleen+Thymus*	–	–	–	1.6±0.1†
Spleen+Thymus*	–	+	–	2.3±0.1
Spleen+Thymus*	–	–	+	2.2±0.1
Spleen+Thymus	+	–	–	1.6±0.09†
Spleen+Thymus	+	+	–	1.6±0.3†
Spleen+Thymus	+	–	+	1.4±0.15†

5×10^6 splenocytes were mixed with 1.25×10^6 thymocytes from controls or mice treated with *C. parvum* (1.4 mg/mouse i.p.) or Pyran (25 mg/kg i.p.). Thymocytes were obtained 2 days after *C. parvum* and 4 days after Pyran.

*Thymocytes obtained from sham-adrenalectomized mice.

†$P<0.05$ vs controls.

DISCUSSION

Data presented show essentially that treatments with *C. parvum*, BCG or Pyran can induce a prompt, marked reduction in thymocyte suppressive activity, as deduced from the inability of thymocytes from treated animals to inhibit a local GVHR mounted by syngeneic spleen cells transplanted in H–2 in-

in support of this hypothesis, although a direct comparison of this study with our results cannot probably be made because higher doses of BCG were used.

Further studies are clearly needed in order to establish whether significant differences exist between these immunomodulators' effects on this specific lymphocyte subset in different lymphoid districts and more work is required

to assess fully the relevance of these changes in the antitumoral activity of these compounds. The observation that pretreatment with antilymphocyte serum abrogates the *C. parvum* antimetastatic effect in mice with Lewis lung carcinoma [26], a system in which tumor growth is conditioned by suppressor T cells [9, 27], is at this time only suggestive, it seems clear, however, that the mechanisms of action of these immunomodulators are more complex than expected.

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Reduced Tumorigenicity by Addition *In Vitro* of Sendai Virus

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Abstract—*Treatment of Ehrlich ascites tumor (EAT) with Sendai virus produced polyploid variants that could grow on selective media, whereas no cell growth occurred in untreated control cultures. All of these variants isolated had more chromosomes and showed less transplantability than EAT.*

The banding patterns of each chromosome were analyzed. The karyotypes of the variants appeared to be more heterogenous than EAT. Other biological properties of cultured control and its variants, agglutinability by concanavalin A and the anchorage dependency of cell growth proved to be unrelated to tumorigenicity. Similar variants were isolated by treating EAT with another cell-fusion inducers, lysolecithin or polyethylene glycol. Like the variants induced by Sendai virus treatment, they had more chromosomes and were less tumorigenic than EAT. However, the frequency of occurrence of these variants ($<10^{-7}$) was far less than that (10^{-5}) of the variants induced by Sendai virus.

It is probable that variants pre-existing in EAT begin to propagate in vitro *after the treatment with fusion-inducing agents.*

INTRODUCTION

IT HAS been shown that morphologically reverted cells are usually derived from cells transformed by exposure to oncogenic viruses or chemical carcinogens. The reverted cells have proved to be revertants or variants lacking some of properties of transformed cells, such as sensitivity to agglutination by plant lectins [1, 2], anchorage dependency of cell growth [3, 4] and tumor-specific transplantation antigens [5, 6]. Most revertants show an increase in chromosome number [7, 8] and a reduced transplantability [5, 9, 10].

We have found by chance that a variant cell appeared in the culture after the treatment of Ehrlich ascites tumor (EAT) with Sendai virus.

This paper reports a study of these cells and the experiments made to try to identify the mechanism of how they were produced.

MATERIALS AND METHODS

Cells

Ehrlich ascites tumor cells were maintained by weekly intraperitoneal transplantation into dd strain of mice.

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Isolation of variants induced by Sendai virus treatment

EAT cells were withdrawn from the peritoneal cavity of tumor-bearing mice who had been inoculated 8–10 days earlier. The tumor cells were suspended in Hanks' balanced salt solution (HBSS) and centrifuged. A suspension containing 5×10^6 to 1×10^7 EAT cells in 1 ml of HBSS was mixed with 1 ml of ultraviolet (u.v.)-inactivated Sendai virus (HVJ, Z strain) at the concentration of 500–1000 hemagglutination units (HAU). This virus was kindly supplied by Dr. M. Homma. The virus-cell mixture was incubated in a water bath at 37°C for 20 min. The treated cells were washed 2 times by centrifugation to remove the unadsorbed viruses. They were then suspended in enriched F12 medium (Nissui Seiyaku), fortified with the amino acids and vitamins of Eagle's MEM, containing 10% calf serum (Flow Lab.) and a reduced amount of sodium bicarbonate (0.05%), were plated in 5×10^5 cell aliquots in 60-mm Falcon plastic dishes. The cultures were incubated in a CO_2-incubator and the medium renewed twice a week. The cultures were terminated within 2 months and the variants were not used for longer than a

month of culture after thawing from the frozen stock for the transplantability testing.

Isolation of variants induced by lysolecithin treatment

Lysolecithin (LL, Sigma Chem.) solution was prepared by the method of Croce *et al.* [11]. EAT cells were centrifuged and resuspended in HBSS.

Then, 5×10^6 cells per 0.5 ml of HBSS were treated with 0.1 ml of the LL solution at final concentration of 4–10 μg/ml. After 1 min of exposure to LL, the LL in the suspension was neutralized by the addition of 0.4 ml of calf serum to each tube, and the treated cells were collected by centrifugation. The cells were resuspended in enriched F12 containing 10% calf serum and 0.05% $NaHCO_3$ and finally 5×10^5 cells were plated into each dish.

Isolation of variants induced by polyethylene glycol treatment

Polyethylene glycol (PEG, 6000, BDH Chem.) solution was prepared as described by Pontecorvo [12].

EAT cells were centrifuged and the supernatant was discarded. Then, 0.3 ml of 50% (w/w) PEG solution was added to the cell pellets. After exposure to PEG for 5 min, the suspension was rapidly diluted by the addition of 5 ml of MEM without serum (serum-free medium) and centrifuged. The treated cells were resuspended in 2 ml of serum-free medium and again centrifuged. The washed cell pellets were resuspended in enriched F12 containing 10% calf serum and 0.05% $NaHCO_3$ and 5×10^5 cells were seeded per dish.

Cell cloning

We cloned the cultured cells using Microtest II plate (Falcon plastic). A cell suspension (0.2 ml) containing 1 cell was placed in each microtiter well. Two weeks after plating, individual colonies were isolated.

Chromosome preparation

Chromosome preparation was made by the air-drying method after colchicine and hypertonic KCl treatment. The slides were stained with Giemsa for routine karyotyping. For the banding pattern analysis, the Giemsa-staining method with trypsin [13] was used.

Transplantability test

The transplantability of various tumor cells isolated was tested by intraperitoneal inoculation into 3–4 weeks old dd strain mice.

The cultured cells were trypsinized and resuspended in 0.2 ml of HBSS containing the cell suspension adjusted to the required cell number. In this case, trypsin did not influence the take rate of these cells, because in some experiments the tumorigenicity of the cells harvested without the treatment of proteolytic enzymes was very similar to those of the cells harvested with the treatment of trypsin. The animals inoculated were observed for 4 weeks and the take incidence was assessed from the number of mice killed by ascitic tumors.

Agglutination by concanavalin A

Agglutination assay for concanavalin A (con A, Sigma Chem.) was carried out by the method described by Burger [14]. The cultures were dispersed with 0.02% EDTA solution and washed 2 times with Ca- and Mg-free phosphate buffered saline. The agglutination reaction was performed in 35-mm Petri dishes with 0.5 ml of cell densities of 10^6 cells/ml and 0.5 ml of a solution with increasing concentrations of con A. After shaking gently for 30 min, the extent of agglutination was measured as the percentage of agglutinated cells. The concentration of con A(μg/ml) required to produce half maximal agglutination was determined.

Growth in medium containing methyl cellulose

The ability of cells to grow in medium containing methyl cellulose was tested according to the procedure of Risser and Pollack [15]. Each of cells was plated in 4 ml of a mixture of 10% calf serum, 1.2% methyl cellulose (Methocel, 4000 counts/sec, Dow Chem.), 90% MEM at the densities of 10^4, 10^3 and 10^2 cells. This mixture was layered onto a plate containing 6 ml of 0.9% agar (Bacto, Difco), 90% MEM and 10% calf serum (Methocel medium). The cultures were fed weekly with 4 ml of the medium.

The colonies were scored after 3 weeks of culture. Only colonies visible to the naked eye were scored.

RESULTS

Culture of EAT cells

EAT cells grew rapidly in MEM or F12 medium supplemented with 10% calf serum.

But almost all EAT cells failed in which the amount of sodium bicarbonate in enriched F12 plus 10% calf serum was reduced from its normal concentration of 1176–500 μg/ml or less. This phenomenon was not observed when MEM plus 10% calf serum was used under the same conditions. Further, EAT cells began to grow *in vitro* when MEM plus 10% calf serum was used without any added bicarbonate in an atmosphere of 5% CO_2 in air.

Detection of Sendai virus (HVJ)-induced variants

We happened to observe a following event: an Ehrlich ascites tumor variant appeared in the culture when EAT cells and human cells were hybridized in the presence of inactivated Sendai virus. These variant cells were then detected in the culture of EAT cells pretreated with Sendai virus on selective medium: a few colonies were found to appear on the plate in which the virus-treated EAT cells were seeded for 1–2 weeks, whereas no such colonies appeared in untreated cultures. This process was repeated about 20 times during 2 yr. In each experiment, these colonies appeared in the virus-treated cultures plated at the densities of 5×10^5 EAT cells/dish. Also, the growing cells were tested for their transplantability. The results showed that the cells were true variants (see Fig. 4).

The frequency of colony formation of these variants was about one per 10^5 EAT cells. Effect of the concentration of Sendai virus on the rate of formation of these variants was investigated: aliquots of 3×10^5 EAT cells were treated with inactivated Sendai virus at titers varying from 10–1000 HAU per tube, and then the treated cells were cultured in the selective medium for 2 weeks. The cultures were harvested, and the numbers of cells were counted. It was difficult to determine the exact rate of colony formation, because the numbers of cells in suspension increased with time. Approximately 3.5×10^5 cells/plate at concentration of 1000 HAU, 0.9×10^5 cells at the 300 HAU and 0.08×10^5 cells at the 100 HAU were counted, respectively. No growing cells were detected at the 30 and 10 HAU. The rates of appearance of true abnormal cells seemed to be dependent on the concentration of the virus. Hence, this virus proved to be a very important factor for the development of these variants.

Chromosome constitution of control cells and HVJ-induced variants

The histogram of chromosome numbers in EAT cells, cultured control cells and HVJ-induced variants is shown in Fig. 1. The histogram in EAT cells showed a major sub-tetraploid peak ranged from 66 to 76 chromosomes per cell with a minor sub-octaploid component ranged from 122 to 151 chromosomes per cell. The distribution pattern of chromosome number in the cultured control was very similar to that of EAT cells. HVJ-induced variants showed a bimodal distribution pattern of chromosome number. But the sub-tetraploid cells had a modal number of 85 chromosome per cell with a considerable scatter and the sub-octaploid cells were distributed in a range from 140 to 170 chromosomes per cell. The representative karyotypes of EAT cell and HVJ-induced variant are shown in Fig. 2.

Comparison of chromosome banding patterns of EAT cells and HVJ-induced variants

The analysis of chromosome banding pattern was carried out on 7 metaphase preparations of EAT cells and on 5 metaphase preparations of their variants. About 63% of the chromosomes in EAT cells and the variants were of normal origin. However, the origin of 19 telocentrics and 7 biarmed chromosomes in EAT cells could not be determined from the banding patterns, whereas 35 different telocentrics and 7 biarmed chromosomes of unknown origin were present in the variants. The results obtained on each metaphase of both cells are summarized in Table 1. Figure 3 shows the marker chromosomes of unknown origin in EAT cells and HVJ-induced variants. There was a striking variation from cell to cell in the range of chromosome types: the number of marker chromosomes which were not seen in any of HVJ-induced variants was 7 in EAT cells, whereas 23 marker chromosomes were present in HVJ-induced variants alone (Table 1).

Transplantability of control cells and HVJ-induced variants.

EAT cells and their variants were inoculated intraperitoneally into weanling mice at various cell densities,. The results of transplantability test showed that 100 EAT cells or 100 cultured control cells killed almost all the animals within 3–4 weeks. Table 2 also indicates that the TFD_{50} values of 5 independently isolated sub-tetraploid clones (clones 1–4) of which the number of chromosomes

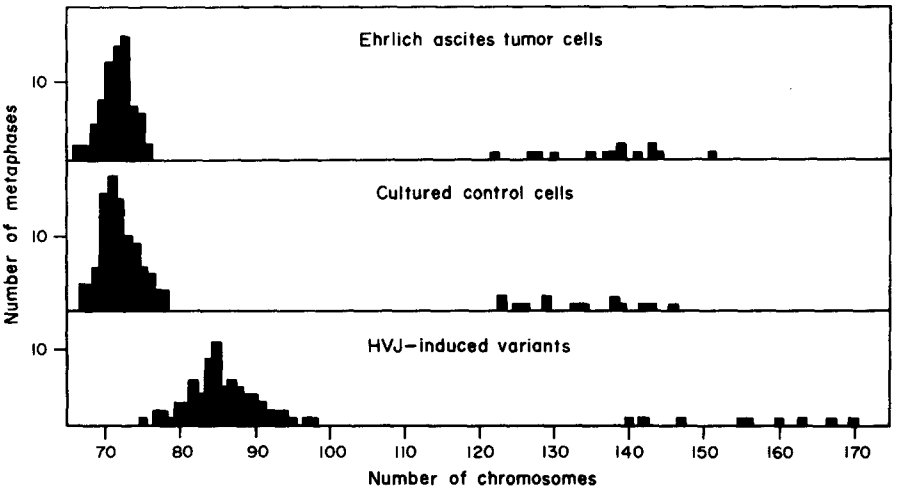

Fig. 1. Distribution of chromosomal number in Ehrlich ascites tumor cells, cultured control cells and HVJ-induced variants. The chromosomal analysis of the mitotic cells in cultured control and variants was performed after three weeks of culture.

ranged from 62 to 74 per cell were similar to those of EAT cells, but the values of 2 sub-octaploid clones (clones 5, 6) were lower than those of the sub-tetraploid lines. The inoculation of 5×10^5 HVJ-induced variants produced a 100% tumor take rate, though the TFD_{50} values were as high as about 10^5 cells as shown in Table 3. This indicates that these variants were approximately 10,000 times less malignant than EAT. Live Sendai virus as well as the inactivated virus had the ability to induce these variants, though with only one tenth the frequency of that induced by the inactivated virus. This suggested that these variants might be converted antigenically as a result of the viral treatment. No viral antigens were detectable by immunofluorescence assay of the variants, even the variants induced by treatment with live virus (K. Sugamura, personal communication). Thus, there was no sign of the synthesis of the viral antigens by the variant cells. To test whether the infectivity of Sendai virus affected the transplantability of the virus-treated cells, each of the variants induced by the live or inactivated virus was inoculated into mice. Table 3 shows that the transplantability of the variants was not influenced by the infectivity of the virus used. Effect of the ploidy of the variants on the transplantability was also compared, 3 sub-tetraploid clones (clones 3–5) to 2 sub-octaploid clones (clones 6, 7), there was no significant difference between them (Table 3). Total percentage of the lethal take in 17 experiments of EAT cells and in 28 experiments of the variants is summarized in Fig. 4.

Effect of cyclic adenosine-3′, 5′-monophosphate on cell growth

Effect of cyclic adenosine −3′, 5′-monophosphate (cAMP) on the growth of cultured control cells and their variants is shown in Fig. 5. The growth of the control was strongly inhibited by the addition of 0.3 mM cAMP (Sigma Chem.) to the medium. At the concentration of 0.05 mM cAMP, some inhibitory effect (about 10% inhibition) was found on the growth of the same cells. When dibutyryl cAMP at the concentration of 0.3 mM was added, it caused less inhibition of growth of the control than cAMP (Fig. 5). However, 0.3 mM cAMP had only slight effect on the growth of HVJ-induced variants.

Agglutination by con A

Cultured control cells were highly agglutinable by con A (less than 25 μg/ml was required for the half-maximal stage to be reached). The cell agglutination of HVJ-induced variants was very similar to that of the control.

Growth in methocel medium

The control cells and HVJ-induced variants after a month of culture were tested for their ability to form colonies in Methocel medium. The results are shown in Table 4. HVJ-induced variants gave 15–22 colonies per 100

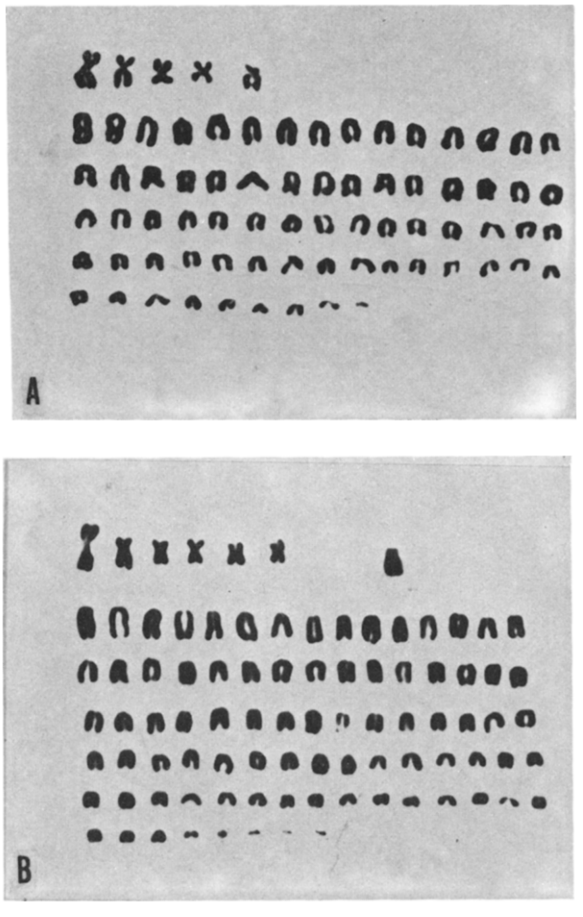

Fig. 2. Karyotypes of Ehrlich ascites tumor cell and HVJ-induced variant. A Ehrlich ascites tumor cell has 74 chromosomes. B HVJ-induced variant has 90 chromosomes.

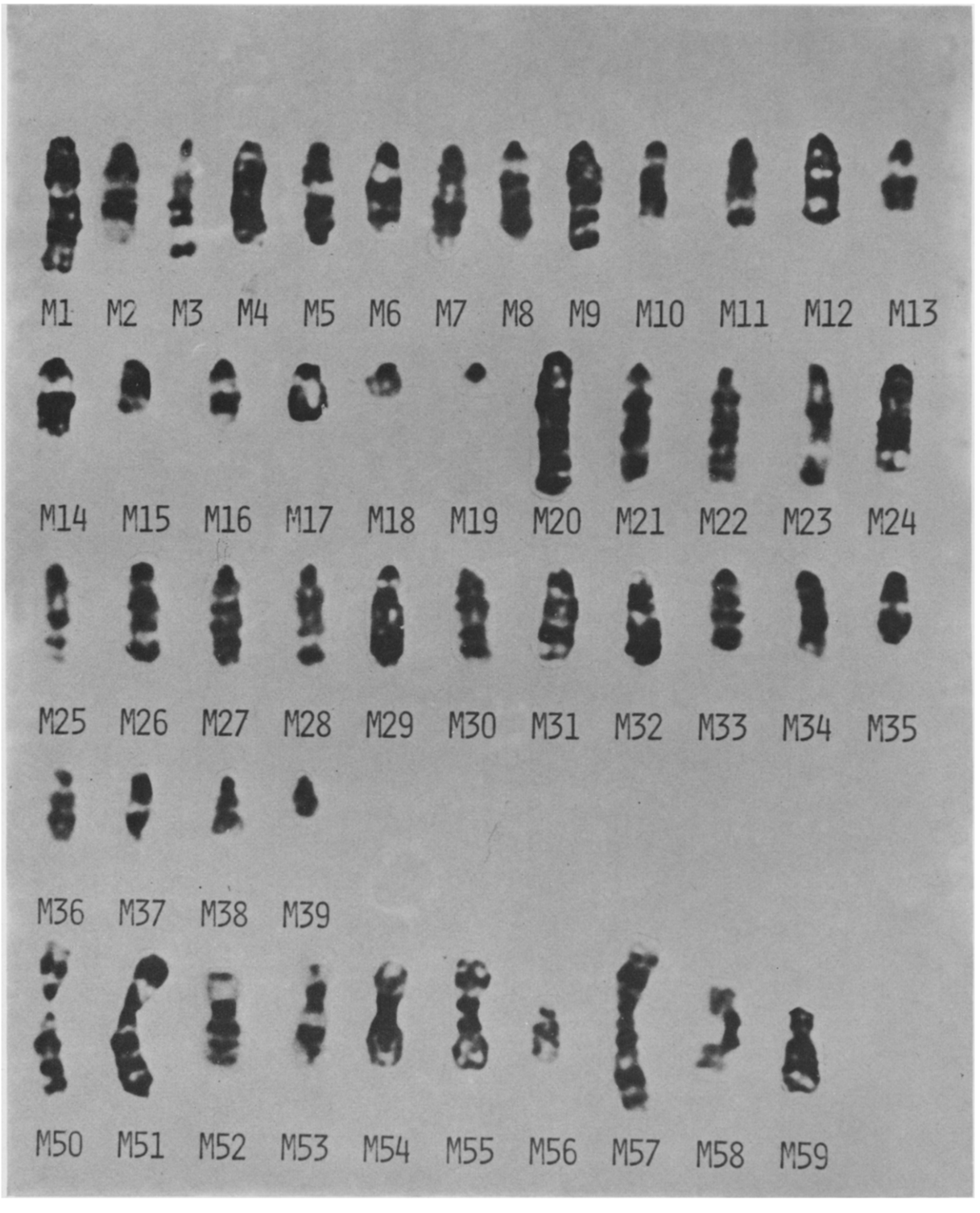

Fig. 3. *Marker chromosomes from Ehrlich ascites tumor cells and HVJ-induced variants. Telocentric markers are designated as* M1 *to* M39 *and biarmed markers as* M50 *to* M59.

Table 1. *Distribution of the number of copies per cell of each abnormal chromosome in Ehrlich ascites tumors and HVJ-induced variants*

Cell type	No. of copies per cell	Marker chromosome M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 13	M 14	M 15	M 16	M 17	M 18	M 19	M 20	M 21	M 22	M 23	M 24	M 25
Ehrlich ascites tumors (7 cells)	0	5			2	1	6			1	5	5	3	1	2	1	2		2	1	7	7	7	7	7	7
	1	2	7	2	4	5	1	4	6	4	1	2	4	1	2	5	4	3		1						
	2			5	1	1		2	1	2	1			4	2		1	3	1	5						
	3							7						1	1	1		1	4							
HVJ-induced variants-2 (5 cells)	0		5	1	4	2	5	1	2	3	5	3	3	2	1	5		3	2	1	1	4	1	4	3	4
	1	4		2	1	3		2	2			2	1	1	1		5	2	1	1	1	4	1	1	1	2	1
	2	1		2				1		1			1	2	2				2	3				3			
	3							1	1	1					1												

Cell type	No. of copies per cell	M 26	M 27	M 28	M 29	M 30	M 31	M 32	M 33	M 34	M 35	M 36	M 37	M 38	M 39	M 50	M 51	M 52	M 53	M 54	M 55	M 56	M 57	M 58	M 59
Ehrlich ascites tumors (7 cells)	0	7	7	7	7	7	7	7	7	7	7	7	7	7	7	5		3	4	1		2	7	7	7
	1															2	6	4	3	4	2	5			
	2																1			2	3					
	3																				2				
HVJ-induced variants-2 (5 cells)	0	1	3	4	4		2	2	2	4		3	4	4	4	4	5	1	5		2	5	1	4	2
	1	2	2		1	3	3	3	2	1	2	2	1	1	1	1		4		5	3		3	1	3
	2	2		1		2			1		3												1		

Table 2. Chromosomal constitution and tumorigenicity of Ehrlich ascites tumor and the cultured original cells

	Cell population	No. of chromosomes				No. of cells inoculated/animal				log TFD_{50}
		Mean	±	S.D.	Range	10^1	10^2	10^3	10^4	
In vivo	Uncloned†	71.8	±	2.7	66– 76	2/3‡	5/5	5/5	5/5	0.75
		146.6	±	12.7	122–152					
	Uncloned†	72.5	±	2.0	67– 78	4/5	5/5	5/5	5/5	
		137.8	±	8.2	124–150					
In vitro	Uncloned†	72.3	±	2.6	68– 80	3/4	5/5	5/5	4/4	0.79
		147.4	±	7.9	134–157					
	Clone-1	62.4	±	2.7	58– 68	2/4	4/4	4/4	NT§	
	Clone-2-1	65.4	±	5.3	54– 76	4/5	3/3	3/3	3/3	
	Clone-2-2	67.0	±	4.1	56– 72	3/4	3/3	3/3	3/3	
	Clone-3	71.0	±	2.6	67– 75	2/3	3/3	4/4	4/4	
	Clone-4	74.0	±	3.9	67– 79	3/4	3/3	4/4	4/4	
	Clone-5	139.2	±	5.8	130–146	NT	1/4	3/4	3/3	2.13
	Clone-6	145.3	±	6.3	130–158	NT	3/4	4/4	4/4	

*Tumor forming dose was calculated by the method of Behrens-Kärber.
†Chromosomes of each cell population were composed of about 70% in tetraploid range and 30% in octaploid range.
‡No. of animals with tumor/No. of animals inoculated.
§Not tested.

Table 3. Chromosomal constitution and tumorigenicity of HVJ-induced variants

Virus treatment	Cell population	No. of chromosomes				No. of cells inoculated/animal					$\log TFD_{50}$*
		Mean	±	S.D.	Range	10^4	5×10^4	10^5	5×10^5	10^6	
None	Uncloned	88.8	±	6.3	78–103	0/3†	NT‡	2/3	3/3	3/3	4.83
	Uncloned	94.5	±	7.6	81–114	0/3	1/3	3/3	3/3	3/3	
	Uncloned	93.0	±	3.7	84–100	0/3	NT	2/3	3/3	3/3	
	Clone-1	87.5	±	2.3	84– 92	0/3	NT	1/3	3/3	3/3	
β-prone inactivated	Uncloned	86.3	±	5.7	80– 96	0/3	NT	2/3	3/3	3/3	4.90
	Clone-2	84.6	±	2.7	81– 89	NT	0/3	1/2	2/2	2/2	
u.v.-inactivated	Uncloned	89.7	±	2.8	85– 94	0/3	NT	1/3	3/3	3/3	4.98
	Uncloned	87.1	±	3.2	81– 94	0/3	NT	1/3	3/3	4/4	
	Uncloned	98.7	±	6.8	88–114	0/4	0/3	2/3	3/3	NT	
	Uncloned	97.6	±	8.1	82–111	0/3	NT	2/3	NT	3/3	
	Clone-3	91.5	±	3.2	87– 97	0/3	NT	1/3	2/2	4/4	
	Clone-4	89.2	±	3.7	83– 99	0/8	2/8	5/8	8/8	NT	
	Clone-5	83.5	±	5.1	76– 95	0/5	NT	3/4	4/4	NT	
	Clone-6	161.9	±	8.4	140–173	0/3	NT	2/4	NT	3/3	
	Clone-7	154.0	±	5.1	143–163	0/3	NT	1/3	NT	3/3	

*Tumor forming dose was calculated by method of Behrens-Kärber.
†No. of animals with tumor/No. of animals inoculated.
‡Not tested.

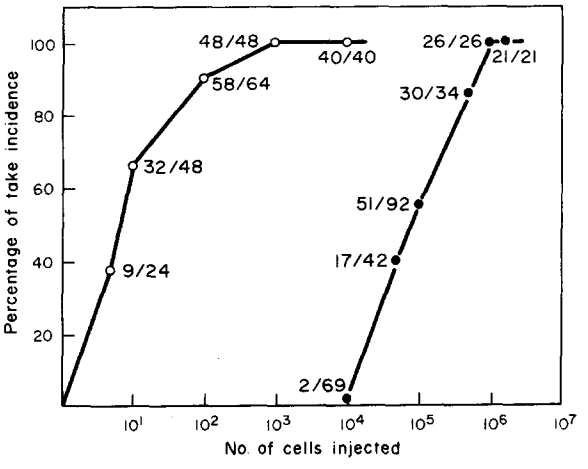

Fig. 4. Correlation between take incidence and number of cells inoculated in EAT cells or HVJ-induced variants. Number in Figure indicates No. of mice with tumor/total No. of mice, and arrow shows the percent tumor. All animals with tumors died.
–○–EAT cells
–●–HVJ-induced variants.

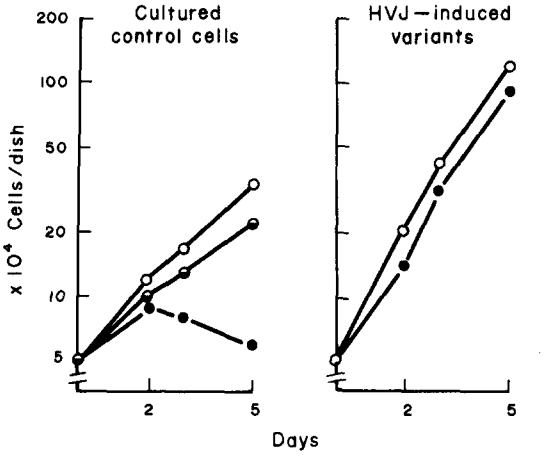

Fig. 5. Effect of cAMP and its derivative on the growth of cultured control cells and HVJ-induced variants. Both cells harvested at days 18 in culture were seeded in MEM with 10% calf serum at the densities of 5×10^4 cells/dish and 0.3mM cAMP or dibutyryl cAMP was added to the medium after 3 hr of incubation. Each point represents the average of two samples.
–○–control.
–●–cAMP.
–◒–dibutyryl cAMP.

cells, whereas the control produced far fewer colonies (0.02–0.08%).

Stability of these biological properties

The stability of the biological properties described above was re-tested in the control and the variants over a year period. The control and the variants obtained by cloning were maintained by the serial passage: these cells at the densities of about 10^5 cells were transferred without trypsinization once a week. The tumorigenicity in both cells was unaltered during the continuous culture *in vitro*. Observation on HVJ-induced variants reveals that the insensitivity of cells to exogenous cAMP and an ability to form colonies in Methocel medium was stable. On the other hand, the instability of these properties in the control cells was demonstrated during 3 months of culture.

When tested at 3 months after plating, the control cells behaved in a different fashion compared to when they were first cultured: the growth of the cells was unaffected by the addition of cAMP and the plating efficiencies in Methocel medium as well as in liquid medium were equal to those of the variants. Thereafter, the control cells were stable over a year period. The results indicated that the stable properties expressed by the control cells were acquired by a sub-set of cells in the original population during 3 months of continuous propagation. There was no correlation between the tumorigenicity and these biological properties. During the serial culture of the variants, we could not find any cells reverted to the control type even through *in vivo* passages.

Attempt to isolate the variants by influenza virus treatment

It is well known that Sendai virus, a parainfluenza virus Type I, possesses many

Table 4. Growth of cultured control cells and HVJ-induced variants in Methocel medium

	No. of experiments	Colony formation in Methocel medium (%)
Cultured control cells	1	0.08
	2	0.02
	3	0.07
HVJ-induced variants	1	18
	2	15
	3	22

biological activities on the envelope of the virion. Our experiment on EAT cells suggest that one of these activities may be related to the occurrence of their HVJ-induced variants. Neuraminidase and hemagglutinating activities are present on the surface of influenza virus [16] as well as Sendai virus [17]. It is, therefore, supposed that variant cells might appear in the culture of EAT cells treated with influenza virus, if any of neuraminidase or hemagglutinating activity was associated with the induction of the variants.

An experiment was made using u.v.-inactivated human influenza virus, PR-8 strain (supplied by Dr. M. Homma). No colonies appeared in the culture, though $>5 \times 10^7$ EAT cells were treated with the virus and cultured.

Attempt to isolate the variants by LL treatment

Sendai virus is known to have high cell-fusing activity on the viral envelope [18]. It is shown from the result of structural analysis of Sendai virus that this virus contains LL in the envelope [19]. LL has an ability to induce cell fusion [20], like Sendai virus. LL is, therefore, expected to have an activity for inducing Ehrlich variants, if the fusion factor was concerned in the induction of the variants. In the repeated experiments, the variants were only detected in the culture of cells treated with 4 or 8 μg/ml of LL. These variants (LL-induced variants) occurred at very low frequency ($<1:10^7$ EAT cells). The frequency was far less than that of HVJ-induced variants.

LL-induced variants, like HVJ-induced variants, had an increased number of chromosomes per cell and a decreased transplantability, as compared with the control (Table 5).

Attempt to isolate the variants by PEG treatment

Recently, it has been reported that PEG can be used to induce cell fusion [12]. We tried to detect the variants from the culture of EAT cells after exposure to PEG. Only 2 colonies arose throughout these experiments. The frequency of colony formation was <1 per 10^7 EAT cells. Table 5 shows that the variants (PEG-induced variants) had more chromosomes and were less tumorigenic than the control. The histogram of chromosome numbers and the TFD_{50} values in LL- or PEG-induced variants showed to be very similar to those of HVJ-induced variants.

DISCUSSION

Ehrlich ascites tumors are highly malignant and have been passaged continuously in any strain of mice for more than seventy years. Therefore, it is probable that the polyploid variants showing less transplantability will be produced spontaneously and accumulated in the EAT population during the continuous passages *in vivo*.

In fact, we isolated many polyploid variant cells with less tumorigenicity from the culture of EAT by treating them with proteolytic enzymes or by modifying the culture conditions, such as low serum-containing medium or high serum albumin-containing medium, though the experiment is still in progress. In the present experiment, HVJ-induced variants appeared in the virus-treated culture of EAT cells at a frequency of one per 10^5 cells. The mechanism by which the variants pre-existing in the population began to propagate *in vitro* after Sendai virus treatment is unknown. Sendai virus possesses several biological activities on the envelope. It includes neuraminidase, hemagglutination [17], hemolyzing and cell-fusing activities [18]. But no such known factors seem to be important for the induction of these variants. Yamada and Hatano [21] have reported that cultured tumor cells persistently infected with Sendai virus have a decreased transplantability compared with the untreated control cells. This phenomenon suggests to be due to antigenic conversion of the tumor cells by the viral infection. However, antigenic conversion of EAT by the virus seem to be ruled out, since there was no evidence of any marker specific for the Sendai virus.

In general, the revertants obtained with the treatment of fluoro-deoxyuridine [8], bromo-deoxyuridine [22], colchicine [23], con A [7], and by plating on glutaraldehyde-fixed monolayers [10] have a higher chromosomes count than the transformed parent cells. The chromosome banding patterns of hyperdiploid EAT have been analyzed by Sasaki *et al.* [24]. They have reported that the cell population is highly heterogenous, and only 30% of the chromosomes are of normal origin, whereas the remaining chromosomes are altered structurally. All of the less tumorigenic Ehrlich variants in our system were characterized by an increase in chromosome number. The analysis of the chromosome banding patterns re-

Table 5. Chromosomal constitution and tumorigenicity of LL- or PEG-induced variants

Agents	Concentration of agents	Cell population	No. of chromosomes				No. of cells inoculated/animal					$\log TFD_{50}$*
			Mean	±	S.D.	Range	10^3	10^4	3×10^4	10^5	3×10^5	
LL	8 μg/ml	Uncloned	93.6	±	3.7	89–100	0/4†	0/4	NT‡	3/4	NT	4.63
	4	Clone	89.6	±	3.6	82– 97	0/4	0/4	NT	4/4	NT	
PEG	50%	Clone	84.9	±	5.8	78–101	NT	NT	0/3	0/3	3/3	5.15

*Tumor forming dose was calculated by the method of Behrens-Kärber.
†No. of animals with tumor/No. of animals inoculated.
‡Not tested.

veals that HVJ-induced variants had a much greater degree of chromosome heterogeneity than EAT. The marker chromosomes of unknown origin were common to both cells. Despite their similarity, the two cell lines were clearly different in the range of chromosome types. For example, 26 different marker chromosomes were present in the control, whereas 42 marker chromosomes were seen in HVJ-induced variants. In addition, each cell in the sample of HVJ-induced variants showed specific variation in the contents of marker chromosomes. Sachs *et al.* [25, 26] have proposed that the genetic control of malignancy in hamster tumor cells and their revertants is controlled by the balance between genes for expression and suppression of malignancy. They have assigned these genes to particular chromosomes. Similarly, specific chromosomes associated with either expression or suppression of malignancy in rodent tumor cells are identified in relation to the balance of these chromosomes [27, 28]. In our system, the suppression of malignancy was associated with an increase of the chromosome number. The analysis of chromosome banding patterns suggests that a group of the marker chromosomes present in HVJ-induced variants alone may be related to the suppression of malignancy. However, it is impossible to assign any specific chromosomes associated with the suppression of malignancy. HVJ-induced variants have more chromosomes ranged from 10 to 20 than the control. Therefore, there is a possibility that the variants might be an artifact resulting from the fusion in culture of EAT cells by the Sendai virus because EAT cells show a high fusion rate when treated with the virus. But the studies of chromosome banding patterns on HVJ-induced variants show that they were true variants pre-existing in their ascites population, since about half of the marker chromosomes in HVJ-induced variants was not seen in any of EAT cells. Yoshida and Funaki [29] have found that hyperdiploid EAT readily grows *in vitro* only after the treatment with Sendai virus, although no cell growth occurs in untreated control. The results obtained by the banding analysis of the chromosomes indicate that 2S cells originated from the hybrids formed between 2 different 1S cells and the cells shifted very rapidly from the 2S to the hyper-1S region. The chromosome constitution of our HVJ-induced variants was stable, and rapid and extensive loss in the chromosome number was not observed during the continuous propagation *in vitro*. In addition, each of LL- or PEG-induced va-

riants derived from EAT was polyploid and had 10–20 more chromosomes than the original EAT, as seen in HVJ-induced variants. The possibility that HVJ-induced variants arose as a result of cell fusion of EAT is unlikely.

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Effect of CCNU on Survival Rate of Objective Remission and Duration of Free Interval in Patients with Malignant Brain Glioma—Final Evaluation*

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Abstract—*The effect of CCNU on three clinical parameters was measured in patients with malignant brain gliomas, operated and treated by radiation therapy* (5500–6000 rad).

From 111 *selected patients included in the first trial* (*No.* 26741) 81 *were avialable for the final evaluation.*

Four questions were answered by this study:

(*a*) *Free interval can be measured in a selected group of patients with malignant brain glioma.*

(*b*) *CCNU* (*mean dosage actually given was over* 100 mg/m^2 *once every* 6 weeks) *does not prolong the free interval.*

(*c*) *Objective remission was observed in* 8 *out of* 27 *patients where this parameter was evaluable. The duration of the remission ranged from* 3 *to over* 14 *months.*

(*d*) *The median survival of patients who received CCNU only after relapse was* 22.5 weeks *from the recurrence and* 62 weeks *from the operation; the figures for patients treated earlier with CCNU were* 11 *and* 43 weeks. *These differences are significant at respectively* 2 *and* 7% *levels.*

Only survival time was measured in the second trial (*No.* 26742), *it was* 31 weeks *in patients treated with CCNU and* 21.5 *in the control group. Although only* 19 *patients were evaluated, the difference is significant at* 1% *level.*

Combined results of both trials suggest so far that: CCNU prolongs the total survival time, produces objective remission in about 30% *of cases and that it should be administered only after relapse, at least in patients with free interval.*

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INTRODUCTION

THIS study, in which we investigate the effects of CCNU on three clinical parameters: total survival time, objective remission and free interval between operation and relapse in patients with malignant brain gliomas, started in January 1973. The last patient was randomized in November 1975. The first evaluation of this investigation has been previously reported [1]. The purpose of this report is the final evaluation.

MATERIALS, METHODS AND STUDY DESIGN

Criteria for patient selection, evaluation of therapy, the design of the two trials and the administration of drugs and radiotherapy have been previously detailed [1, 2]. One hundred and eleven patients were entered into the first trial (26741). All patients had an

optimal resection of a histologically proven malignant glioma, three weeks prior to randomization for chemotherapy. Patients selected for this trial had minimal postoperative neurological deficit and did not require the administration of steroids. All patients were treated with 5500–6000 rad. After randomization, group I (early CCNU) received CCNU 130 mg/m^2 every 6 weeks whereas group II (late CCNU) remained without chemotherapy until the recurrence. After recurrence (end of the free interval) all patients received CCNU. The main clinical characteristics of the patients from groups I and II are compared in Table 1.

Trial 26741

The effect of CCNU on the free interval was evaluated in 81 patients. As shown in Fig. 1 the curves of the free interval duration for patients receiving CCNU and for the controls are very similar. The mean duration of the free interval being 34.5 weeks in patients treated with CCNU and 31.0 in the controls. The mean dosage of CCNU actually given was at least 100 mg/m^2 of the scheduled dose. When the free interval was considered in the whole group of 81 patients regardless of chemotherapy, it appeared longer in patients under 50 yr (median duration 60.2 weeks) than in the older group (28.6 weeks) Fig. 2. This interval tended to be shorter in patients with glioma multiforme (29 weeks) as compared with other pathological types (38 weeks), but the difference is not statistically significant. No differences in the length of the free interval were found by comparing: (a) males to females, (b) apparently total tumor resections to other neurosurgical procedures, (c) patients with neoplastic involvement of one lobe to those with lesions extending to more than one lobe.

Objective remission, defined as a clear cut clinical improvement lasting at least 6 weeks after a complete discontinuation of steroids was evaluable in 27 patients who received at least three courses of CCNU after the relapse. Such an objective improvement was observed in 8 cases (29%), its duration ranged from 3 to 14(+) months (median 7(+) months).

Table 1. *Distribution according to possible prognosis factors*

		Group I (early CCNU)	Group II (late CCNU)
Age	under 50 yr	22	24
	over 50 yr	33	28
		(4)	
Sex	male	41	30
	female	18	22
Pathology	glioblastoma multiforme	24	20
	astrocytoma grades III and IV	15	19
	other	20	13
Type of resection	biopsy	3	2
	partial	20	22
	total	22	17
	Other	14	11
Lobes involved	one	27	34
	more than one	32	18

Only 23 patients were entered in the second trial (26742). All of them required the administration of steroids after surgical removal of malignant glioma and their neurological examination was difficult to evaluate quantitatively. All patients were treated with 5500 to 6000 rad. After randomization 10 patients received CCNU 130 mg/m^2 every 6 weeks, 13 remained without chemotherapy. In this trial, the effect of CCNU was evaluated only with respect to the duration of the postoperative survival time.

RESULTS

The results of the two concomitant studies 26741 and 26742 will be considered separately.

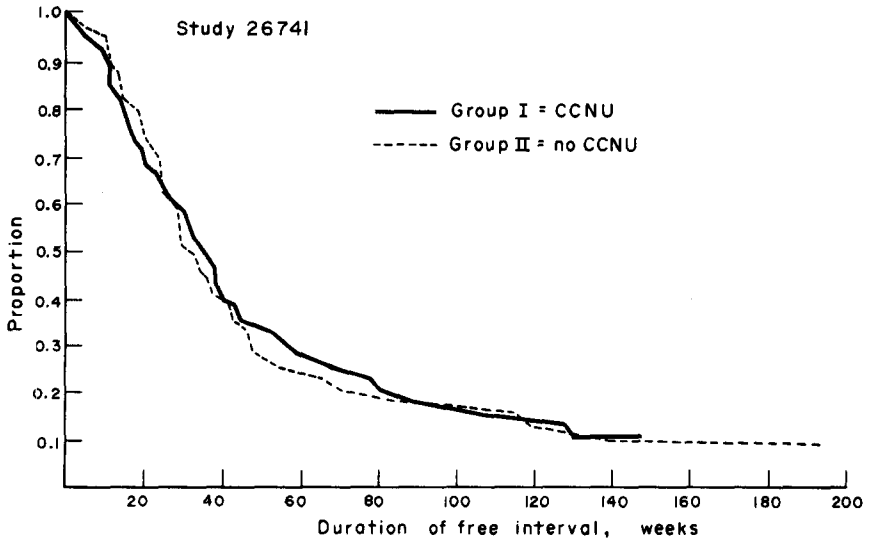

Fig. 1. Study 26741. Free interval length curves in 42 patients receiving CCNU (———) and 39 controls (– – –).

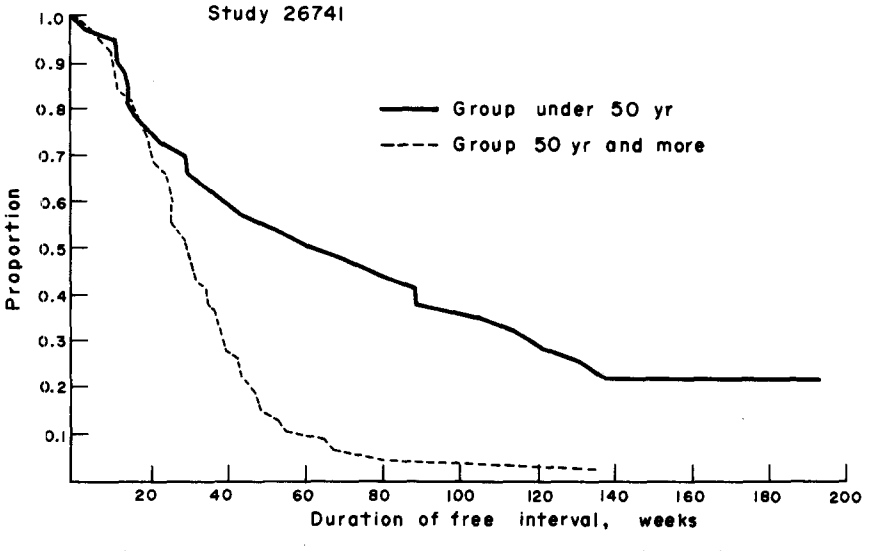

Fig. 2. Study 26741. Free interval length curves in 33 patients under 50 (———) and 48 patients aged 50 yr or more (– – –).

The median survival of patients who received CCNU only after relapse (group II) was 22.5 weeks from the recurrence and 62 weeks from the operation, the figures for patients treated with early CCNU (3 weeks after neurosurgery) were 11 and 43 weeks, the differences being significant at respectively 2 and 7% levels (Fig. 3). Total survival times tended to be longer in patients under 50 yr (63 weeks) as compared to the group of older patients (43 weeks) and in patients with astrocytome grades III and IV (56 weeks) as compared to glioma multiforme (43 weeks) but the differences were not statistically significant. The length of the postoperative survival was not related to sex, type of neurosurgical procedure or number of lobes involved by the tumor.

Severe hematological toxicity characterized by platelets under 60,000 per mm^3, leucocytes under 2500 per mm^3 and hemoglobin level under 10 g/100 ml were observed in respectively 13, 12 and 5 patients from group I and 8 and 4 and 4 patients from group II. Alteration of the liver test was observed in 2 cases. No death was attributed to chemotherapy.

Trial 26742

The median survival was significantly longer in patients who were treated with CCNU (31 weeks) when compared to controls not treated by chemotherapy (21.5 weeks), the difference is significant at the 1% level.

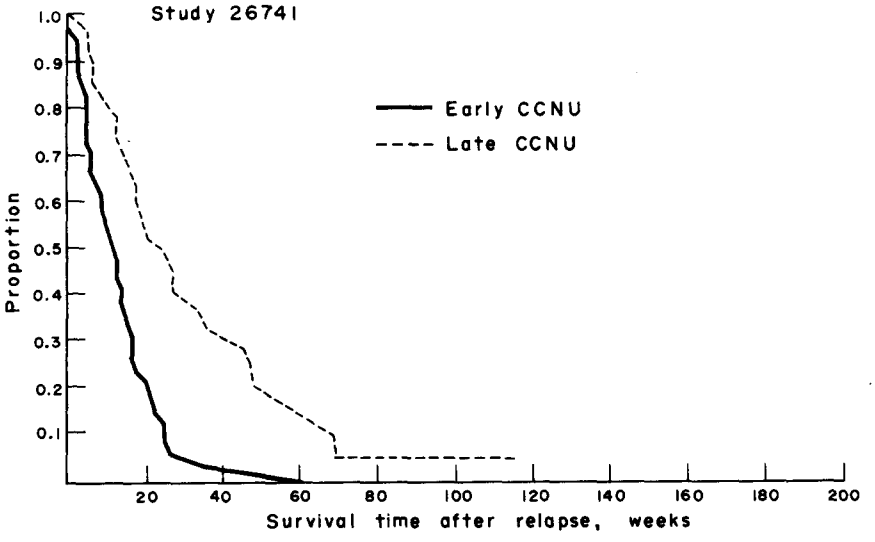

Fig. 3. Study 26741. Survival time curves after relapse in 24 patients treated with CCNU 3 weeks after neurosurgery (———) and in 34 patients treated with CCNU only after relapse (----).

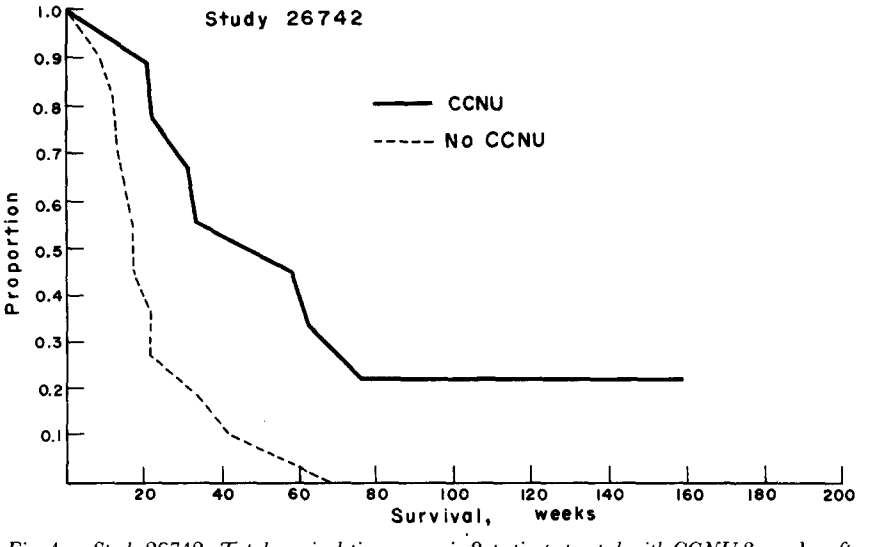

Fig. 4. Study 26742. Total survival time curves in 8 patients treated with CCNU 3 weeks after neurosurgery until death (———) as compared to 11 controls who received no chemotherapy (----).

DISCUSSION

This final evaluation of the two trials confirms the conclusions of the first report of our results concerning the use of CCNU in malignant brain gliomas [1]:

(a) In patients with clinically evaluable free interval the relapse of neurological signs is not delayed by CCNU (trial 26741).

(b) Objective remissions may be expected in about 30% of patients with recurring neurological signs due to malignant brain gliomas after treatment with CCNU (trial 26741).

(c) In a selected group of patients receiving CCNU after neurosurgery, CCNU prolongs the survival time (trial 26742).

How do these results compare with the data from the literature?

To the best of our knowledge, the effects of CCNU on the free interval (median time to progression) were reported in only one study where the free interval was found to be prolonged in patients who received CCNU [3]. The discrepancy between those results and our data are difficult to explain. Scheduled doses of CCNU were the same in both studies. In our trial no patients received dexamethazone before recurrence and the

number of cases studied was larger than in the report of Band *et al.* [3].

The mean dose of CCNU actually given in the present study was over 100 mg/m^2 once every six weeks, which represents 80% of the scheduled dosage. Blood-toxicity was the major limiting factor in patients who received less than the scheduled dosage. It is thus unlikely that, with the schedule used in this study, substantially higher doses of CCNU could have been given during mean periods of about 30 weeks.

Objective remissions after administration of the 3 nitrosourea derivatives currently used: BCNU, CCNU and methyl CCNU have been observed by various authors [4–7], BCNU being possibly slightly superior to the two others. The percentage of objective remissions observed in this study was lower than the figures reported for CCNU by Rosenberg *et al.* [5] and Wilson *et al.* [7], but comparable to the results of Reagan *et al.* [8]. These variations are due at least partially to differences in the definition of objective remission which includes in some studies a stabilization of the neurological status whereas in other trials, including ours, objective improvements were required. The length of objective remission due to CCNU found in this study is in agreement with the results of Wilson *et al.* [7]. Objective remissions were observed in the group of patients who received CCNU only after recurrence. This group of patients had a longer survival from the operation and from the relapse of neurological signs as compared with the group of patients treated with CCNU early after neurosurgery. Therefore, we should recommend that CCNU, when used alone, should be given only after the relapse of neurological signs. The failure of CCNU to prolong the free interval is not inconsistent with its ability to produce objective remissions. The two parameters may not measure the same phenomenon. One may speculate that patients with free interval are predominantly those with a slow postoperative tumor growth rate, possibly due to radiation therapy. Thus, early administration of CCNU will have little antitumor effect, but may select cells resistant to the drug. For the same reason, tumors of patients treated with CCNU only after recurrence may be more sensitive to the drug. In addition, because of the cumulative toxicity of the nitrosoureas, these patients can be treated more vigorously than those who received CCNU immediately after operation.

From various possible prognostic factors, evaluated in the present study which may affect the length of the free interval and the survival time, patients age and tumor pathology appeared as the most important. Younger patients survived longer and glioma multiforme had a worse prognosis than other malignant gliomas. Surprisingly the number of lobes involved by the tumor and the type and extent of the tumor resection were not correlated with the duration of the free interval or the postoperative survival.

The finding that in trial 26742 the survival was significantly longer in patients treated with CCNU as compared to the controls who did not receive chemotherapy at all needs future confirmation. Indeed the number of patients included into the trial is small and its results conflict with the findings of Reagan *et al.* [8] who failed to show in a randomized study a significant increase of the survival when CCNU was added to neurosurgery and radiation therapy.

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A Comparison of the Effect of Metal–Bleomycin Complexes on the DNA Damage and Survival of Cultured Mammalian Cells

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Abstract—*Parallel studies of DNA strand breakage and colony forming ability were carried out on cultured Chinese hamster V79 cells treated with both metal free bleomycin and the copper and cobalt complexes of this antineoplastic agent.*

It has been shown that Cobalt (III) *bleomycin is non-toxic towards cultured mammalian cells and, with the system used, does not cause any detectable DNA damage even though its binding to DNA is enhanced compared to other bleomycins. The effects of Copper*(II) *bleomycin on intact cells is similar to metal free bleomycin with regard to both cytotoxicity and DNA damage. The difference between these two metal complexes is explained by their different stabilities in the cells.*

INTRODUCTION

THE ABILITY of the bleomycin group of antineoplastic agents to bind metals and localise in tumours has been exploited in the field of nuclear medicine. The incorporation of appropriate radioactive metals into the bleomycin molecule has produced a variety of compounds useful for the imaging of tumours [1]. The importance of this metal binding capacity in chemotherapy with bleomycin is as yet unclear.

A likely mechanism for the efficacy of bleomycin in the treatment of cancer is its reported attack on DNA [2], but the role of metal ions in this attack is complex. In initial *in vitro* experiments with DNA, attack was only observed after the addition of sulphydryl compounds to the reaction mixture [3]. It was subsequently demonstrated that DNA attack could be observed *in vitro* in the absence of such sulphydryl compounds if more sensitive assay procedures were employed [4], indicating that such compounds merely enhanced DNA attack. It has been shown that under aerobic conditions Fe^{2+} enhances bleomycin attack on DNA in the absence of sulphydryl compounds [5] and a mechanism has been proposed [6]. Enhancement of attack has also been demonstrated for hydrogen peroxide [7] and the superoxide ion [8] but the addition of EDTA had no effect [9] or inhibited attack even in the presence of sulphydryl compounds [10]. The enhancement of bleomycin attack on DNA produced by sulphydryl compounds has been found to be inhibited by Cu^{2+}, Co^{2+} and Zn^{2+} [5].

It has been suggested that the role of the sulphydryl compounds is to compete with the bleomycin for metals that would normally interact with it and inhibit its attack on DNA [5, 11].

In the clinical situation it was thought that copper would be the metal most likely to inhibit the efficacy of bleomycin due to its abundance in the blood. Preliminary results with patients of a protocol designed to lower serum copper levels during bleomycin therapy are encouraging but assessment of the results is still in progress [12].

In order to understand and improve treatment with bleomycin it is necessary to determine the effect of metals on their action *in vivo*. We report here the effect of preformed metal–bleomycin complexes on the colony forming ability of cultured mammalian cells and the DNA damage produced in these cells.

MATERIALS AND METHODS

Bleomycin sulphate for injection (Lot 259) was obtained as a freeze-dried preparation

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in 15 mg ampoules (containing 15 units of activity) from Lundbeck Ltd., Luton. Beds. Due to differences in potency between the various bleomycins and slight changes in their ratios in the clinical preparation the ampoules contained only ~8.3 mg (wt) of bleomycin.

The cobalt III and copper II complexes were made by adding an equimolar amount of cobalt II or copper II dissolved in water (calculated assuming an average bleomycin mol. wt of 1425) to a weighed amount of bleomycin also in water. The pH was then adjusted to ~pH 7.5 with 0.1M $NaHCO_3$. This procedure allowed complete oxidation of the cobalt II to cobalt III when bound to the bleomycin. Details of the characterisation of metal–bleomycin complexes have appeared elsewhere [13–16].

Cell culture and treatment

Chinese hamster V79 cells were chosen for this study. They have a doubling time of 12 hr and from single cells produce colonies suitable for counting by naked eye within 6–7 days. The growth medium used consisted of Eagle's Minimum Essential Medium with Earle's Salts, plus 15% foetal calf serum. The pH of the medium during subculture and colony growth was controlled by adding 2.2 g $litre^{-1}$ of sodium bicarbonate and maintaining a 5% CO_2 atmosphere in the 37°C incubator. Throughout the course of these experiments the cells were grown as surface-attached monolayer cultures and subcultured whilst still in exponential growth.

Cells to be treated with the bleomycins were plated two days before exposure to the drug. Routinely, 20, 5 cm plastic Petri dishes were seeded from the same stock culture bottle with approximately 5×10^4 cells per dish, yielding $\sim 5 \times 10^5$ cells per dish at the end of the two day growth period. After one day, half of the cell samples were radioactively labelled with 0.02 μCi ml^{-1} of ^{3}H-thymidine (The Radiochemical Centre, Amersham. TRK418, 40–60 Ci $mmole^{-1}$). The level of activity was chosen to minimise the damage to DNA produced by the radioactive label itself (see results section for data). Exposure to the ^{3}H-thymidine was maintained for 15–16 hr at the end of which period the labelled medium was aspirated out of the culture dishes. Each cell sample was washed twice with 5 ml of label-free medium, after which the cells were grown for one hour in medium free of ^{3}H-thymidine before treatment with bleomycin. The same washing procedure was also carried out with the unlabelled cell samples so that as far as possible both sets of cell samples were exposed to the same conditions.

The cell samples were exposed to bleomycin for one hour whilst still attached to the Petri-dish. Small volumes of the bleomycin solution were injected directly into the full growth medium above the cells and mixed by gentle rocking of the dishes. During treatment the cell samples were kept in a 37°C incubator, which was humidified and gassed with a 5% CO_2 in air, gas mixture. At the end of the treatment period the bleomycin containing medium was aspirated out of the culture dishes and each cell sample was washed once with 3 ml of fresh medium and once with 2.5 ml of 0.1% trypsin made up in 0.9% NaCl. The cells were then incubated for 10–20 min at 37°C with 0.3 ml of the trypsin solution in each dish in order to detach the cells from the surface. At the end of trypsinisation each sample was suspended in 5 ml of culture medium which was then repeatedly drawn up into a narrow tipped pasteur pipette to produce a single cell suspension.

The labelled cells were used to assay for DNA damage and the unlabelled cells were diluted and re-plated to assess their clonogenic forming ability, which for the basis of these studies was the criterion used to define cell survival. A cell count was made with a haemocytometer for each cell sample treated so that the survival rate could be expressed in terms of the number of cells plated and then corrected for plating efficiency from the survival rate of untreated control cells. The colonies were stained with methylene blue after 6 or 7 days of incubation.

DNA damage assay

The method of treating the cells to unwind the DNA was similar to that described in detail by Rydberg [17] and was as follows.

Three one millilitre aliquots of each cell suspension were placed in ice-cold polystyrene centrifuge tubes and spun at 1000 rev/min for 5 min using an angle head. (Cat. No. 6885) in an MSE 6L centrifuge maintained at 4°C. The serum was separated from the sedimented cells by inverting each tube and lightly blotting the open end with absorbent paper. The tubes were then placed in an ice bath and 0.5 ml of the lysing

solution (0.03M NaOH, 1.0M NaCl, 0.5% Sarkosyl) was added to each, taking care not to disturb the tubes once the lysing solution had been added. This is the most sensitive step in the procedure [17]. After 15 min, during which they were protected from light, the solutions were neutralized by rapidly adding 1.1 ml 0.02M $NaHPO_4$ using a syringe and needle. Each sample was then sonicated for ten seconds using an MSE sonicator fitted with a 3 mm tip and set at 15 μm amplitude. After sonication 0.2 ml of a 2% SDS solution was added to each solution whereupon they were stored at 4°C for one to three days until chromatography was performed.

The hydroxyapatite was prepared in this laboratory using a' method described by Myazawa and Thomas [18]. The columns were made from the barrels of 2 ml polystyrene syringes, with glass fibre discs to retain the hydroxyapatite (millipore Cat. No. AP2001000) and 3.8 cm, 19 gauge needles to stabilize the flow. Each column contained 0.6 ml of settled hydroxyapatite, was maintained at 60°C in a heated aluminium block during use, and was stored in water at room temperature. Each column could be used for up to five separate determinations.

The chromatography of the DNA was performed in basically the same manner as described by Rydberg [17]. Before use each column was washed with 3 ml 0.5 M and 0.0125 M potassium phosphate buffer, pH 6.8. The ~1.5 ml samples were then loaded, eluted and followed by 3 ml 0.0125 M phosphate buffer. This 4.5 ml sample constituted the run through and the counts it contained gave a sensitive indication of the separation efficiency (see Results). The columns were then washed with 3 ml 0.125 M phosphate buffer, to elute the single-stranded DNA, followed by 3 ml 0.5 M phosphate buffer to elute the double-stranded DNA.

After use the columns were washed with 3 ml 0.5 M and 0.0125 M phosphate buffer prior to storage or reuse. The radioactivity in the fractions was measured in a Beckmann Scintillation Counter using 5 ml of scintillant consisting of 1.1 litres of triton X-100 mixed with 0.9 litres of xylene containing 0.3% PPO and 0.01% POPOP.

RESULTS

The technique of hydroxyapatite chromatography developed by Ahnstrom and Erixon [19] and studied in detail by Rydberg [17], was used to determine the amount of DNA damage in the cells by the bleomycin as it is better suited to the analysis of large numbers of samples than gradient sedimentation. Strand scission is measured by the percentage counts in the single and double stranded fraction of the DNA after unwinding. Initial experiments were performed on untreated cells to determine the optimum amount of ^{3}H-thymidine required to label the DNA, and the time of lysis. In agreement with Rydberg [17] the rate of unwinding was found to be linear with time. With 30 min lysis the highest background double stranded values (Fig. 1) were

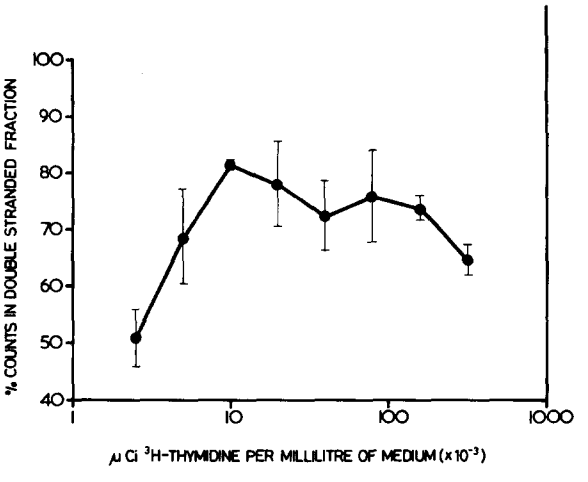

Fig. 1. The effect of the amount of ^{3}H-thymidine used to label the cells on their background DNA damage.

obtained when using a concentration of $\sim 2 \times 10^{-2}$ μCi ^{3}H-thymidine per millilitre of the medium ($\sim 10^5$ cells) and this concentration of label was used throughout the course of the experiments.

During the experiments an empirical rule was developed such that the results of an individual DNA-damage determination were rejected if the counts in the "run-through" fraction of that determination exceeded 0.5% of the total. This rule was developed because the results of determinations having high run-through counts invariably fell well outside the standard deviation of the remaining pair in each triplet and because this figure was high in experiments which had known defects in technique.

The cell survival and DNA damage studies were performed in parallel. Each study was performed on three or more occasions for each bleomycin formulation. The results are plotted as the means and standard deviations

of all the runs; as surviving fraction vs dose of bleomycin (in units of potency) in Fig. 2 and percentage counts in the double-stranded DNA fractions vs dose of bleomycin in Fig. 3. Points without error bars occur where studies for that dose were only made on one occasion. The percentage of counts in the double stranded fraction of the untreated cells was 93.6 ± 2.04 (10 studies) but for clarity the results in Fig. 3 are normalised to 95% background levels.

With the experimental conditions employed significant deviation from "background DNA damage" in untreated cells was not detectable until concentrations of ~ 0.1 unit bleomycin ml^{-1} were employed at which point cell survival was between 1 and 10%. This may be due to repair of DNA damage and/or lack of sensitivity of the system. (The latter could be increased by altering the lysing conditions see Rydberg [17].

Higher concentration of cobalt bleomycin showed increased double stranded counts over the untreated cells which may be an artefact caused by the binding of cobalt bleomycin to DNA interfering with the unwinding process in the alkaline lysing solution.

The cell survival data are expressed as per cent survival of untreated controls. There was no correlation between response to bleomycin treatment and the plating efficiency for untreated cells, which varied between 50–75% during the course of these experiments. The sensitivity of the V79 cells to metal free bleomycin is similar to that reported previously, with a relatively rapid initial response for drug concentrations up to 0.05 units ml^{-1} followed by a reduced rate of response with increasing concentration.

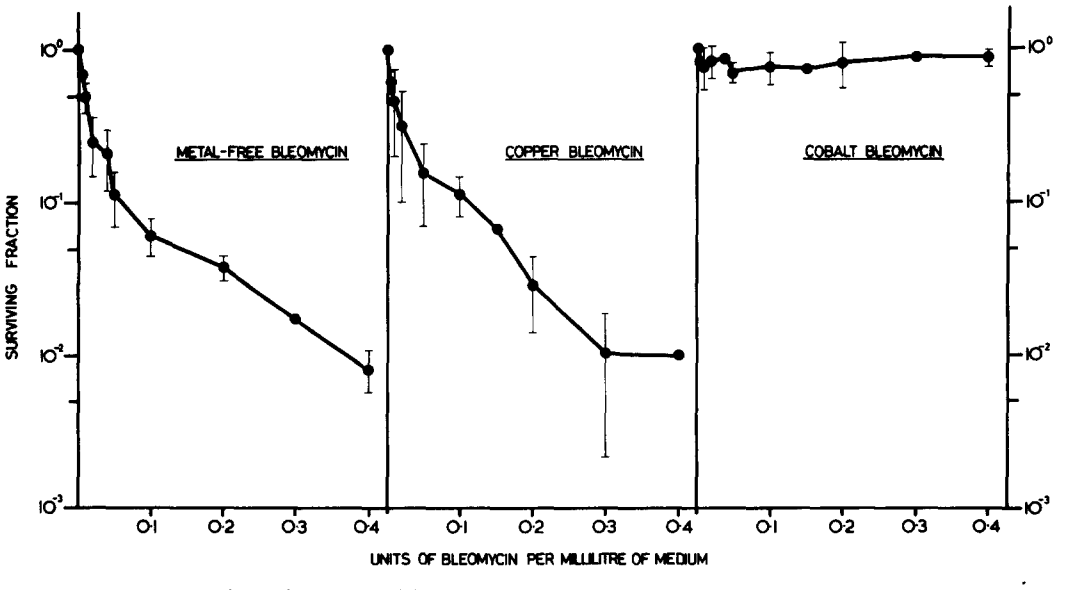

Fig. 2. The effect of the dose of bleomycin on the surviving fraction of cells exposed for one hour.

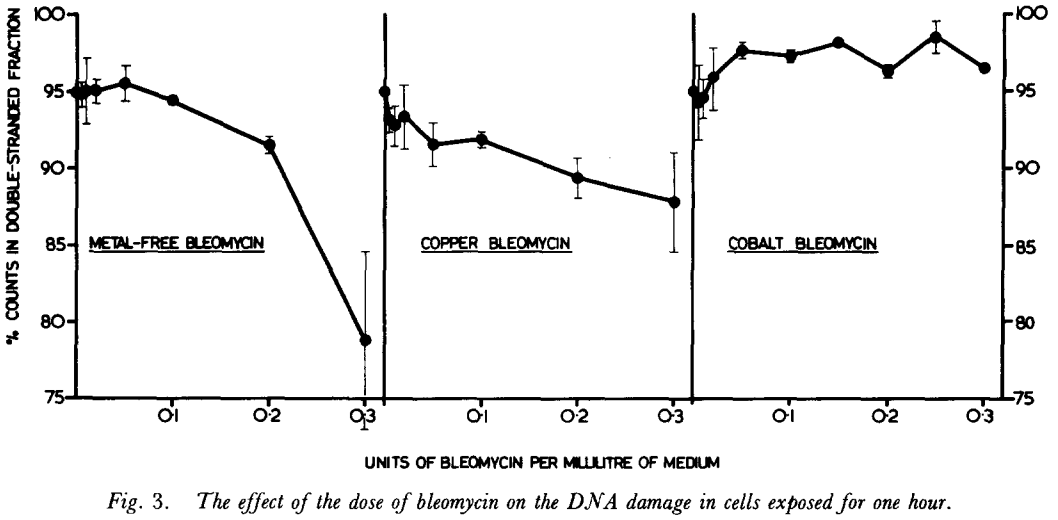

Fig. 3. The effect of the dose of bleomycin on the DNA damage in cells exposed for one hour.

The copper bleomycin survival curve was not significantly different from the curve obtained for metal free bleomycin. In sharp contrast to these two results the cobalt bleomycin was found to be relatively non-toxic for concentrations up to 0.4 units ml^{-1}, which was the highest level investigated.

DISCUSSION

It has frequently been demonstrated that *in vitro* DNA is attacked by bleomycin in the absence of 2-mercapto ethanol or other sulphydryl compounds [4, 6, 7]. It is also known that sulphydryl compounds, on their own, can attack DNA and cause single strand scission [7, 20, 21]. It is not clear whether *in vitro* bleomycin enhances DNA attack by sulphydryl compounds or vice versa.

Understanding of the situation is complicated by the fact that the mechanism involved in the attack of DNA by both bleomycin and sulphydryl compounds is probably the same. Lown *et al.* [7] have recently proposed a mechanism for bleomycin whereby free radials are produced by interaction between bleomycin, FeII and O_2. Wilson [22] describes an identical reaction between sulphydryl compounds, FeII and O_2 which is presumably responsible for the DNA attack attributed to sulphydryl compounds by various authors [7, 20, 21].

Cobalt II and Copper II react rapidly with bleomycin under approximately physiological conditions of pH and ionic strength to produce stable chelates [15]. Numerous other metals have been shown to bind relatively strongly to bleomycin and the complexes of some have been partly characterised [13, 14, 15]. Difficulty has been encountered in producing stable FeII and FeIII bleomycins [15, 22, 23] and Sausville *et al.* [6] have shown that FeII forms a complex that is moderately stable only in the presence of oxygen. Metals such as calcium and magnesium form relatively weak, non chelate complexes with bleomycin.

Binding of bleomycin to DNA *in vitro* occurs with or without the presence of sulphydryl compounds but it is inhibited in the presence of CuII, ZnII or EDTA [24]. Conversely, cobalt bleomycin has been found to concentrate in tumours to a greater extent than other bleomycins and enters the cells of the tumour to become localised in the nucleus, where it is firmly bound to DNA [1, 25]. Using gel filtration we have found that cobalt bleomycin has a greater affinity for calf thymus DNA than has metal-free bleomycin, in contrast to the inhibitory effect, on DNA binding of the metals noted above.

If the cytotoxic action of bleomycin is directly related to attack on DNA demonstrated *in vitro* one would expect that there would be no loss of viability in cells exposed to cobalt or copper bleomycin. Whereas this is true for cobalt bleomycin it is clearly not the case for copper bleomycin. Our results show that copper bleomycin and metal-free bleomycin produce survival curves which are almost identical and which are the normal downward concave type previously reported for mammalian cells [26].

The inhibitory role of cobalt in the attack of DNA by bleomycin *in vitro* is confirmed by the results reported here of the assay for DNA damage in intact cells when treated with cobalt bleomycin. Copper bleomycin and metal-free bleomycin produce similar DNA damage curves which are dose dependent. Thus cobalt bleomycin is not cytotoxic and, with the system used, does not produce any measurable DNA damage. On the other hand the effect of copper bleomycin on intact cells is similar to metal-free bleomycin with regard to both cytotoxicity and DNA damage.

The difference between cobalt bleomycin and copper bleomycin when acting on whole cells compared with their similarity of action on isolated DNA must be related to the greater complexity of the chemical environment in the cells.

Cobalt bleomycin is inert because the electron configuation of the Cobalt III is d^6 [27] and so the active site of the bleomycin is blocked both *in vitro* and in the cells. However, we have found using ^{61}Cu that the copper in copper bleomycin is labile under approximately physiological conditions of pH and ionic strength and without added reducing agents. the labile nature of the copper in copper bleomycin together with the presence of other possibly stronger copper binding ligands in the cell means that competition can occur for the copper, leaving the bleomycin free to attack the DNA if the competition is effective.

Recently Takahashi *et al.* [28] have also reported that copper bleomycin causes scission of DNA *in vivo* even though previous *in vitro* studies had shown that copper inhibits DNA attack by bleomycin. These authors

demonstrated that homogenates of rat ascites hepatoma AH66 cells contained small molecular weight substances that reduced the cupric ion of the bleomycin copper complex. This resulted in transfer of copper from the complex to cellular proteins, leaving metal-free bleomycin to attack the DNA of the cell. Our results are consistent with these findings.

The results reported here suggest that the original idea behind the penicillamine protocol to lower serum copper levels [12] may be invalid as copper does not affect the toxicity of bleomycin towards cells. Although assessment of the results is not yet complete the protocol does appear to have some benefit for patients treated with bleomycin. This may arise because the formation of the copper chelate in the blood affects the subsequent distribution and excretion of the bleomycin [25]. Alternatively the protocol may be affecting the tissue concentrations of other more critical metals; certainly serum zinc levels are known to change as a result of bleomycin administration [29].

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In Vitro Effects of Oestrogen on Testosterone Metabolism by Human Breast Cancers

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Abstract—*The effect of* in vitro *addition of oestradiol on the metabolism of* $7\alpha^3H$ *testosterone by 15 human breast cancers has been investigated. Oestradiol significantly inhibited metabolism and conversion to 5α dihydrotestosterone, 5α androstanediol and Δ4 androstenedione.*

INTRODUCTION

HUMAN breast cancers can metabolise steroid precursors to active hormones such as 5α dihydrotestosterone [1, 2] and oestradiol-17β [3, 4]. Whilst the metabolism of testosterone by rat mammary tumours has been shown to be influenced by oestrogen both *in vitro* [5] and *in vivo* [6], little is known about the effects of hormones on steroidogenesis in human tumours. The aim of the present study was to determine *in vitro* the effect of oestradiol-17β on testosterone metabolism by human breast cancer.

MATERIALS AND METHODS

Tumours from 15 patients with proven cancer of the breast were studied: 10 subjects were at least 5 yr postmenopausal, 4 were premenopausal and had regular menstrual periods at the time of investigation. One patient had undergone bilateral oophorectomy 15 months previously.

Following excision the tumours, 10 of which were primary carcinomas and 5 recurrence on the chest wall, were processed at 0°C. They were finely sliced and split into duplicate portions each weighing 0.5 g to which Krebs Ringer phosphate buffer pH 7.4 (5 ml), an NADPH generating system and 20 μCi $7\alpha^3H$ testosterone (5.7 Ci/mmole from Radiochemical Centre, Amersham) were added.

Incubation was then carried out either in the presence of oestradiol (1.5 μg/ml) or its vehicle (propyleneglycol:ethanol 1:1) by shaking at 37°C in an atmosphere of oxygen for two hours. In two tumours oestradiol-3-methyl ether (1.5 μg/ml) was added to a further incubate as an additional control. The reaction was stopped by the addition of methanol (40 ml) and the incubate stored at −10°C.

Before extracting the metabolites, 500 μg each of non-radioactive testosterone, 5α dihydrotestosterone (5α DHT), 5α androstanediol, Δ4 androstenedione and oestradiol-17β were added to each incubate to monitor recovery losses. Details of the methodology for steroid purification by thin layer chromatography and characterisation by chemical derivative formation have been described previously [7].

Metabolism of testosterone was determined by measuring the incorporation of radioactive label into the appropriate metabolites after correction for recovery losses. Statistical comparisons between the results of incubates with and without added oestradiol were performed by paired *t*-tests after logarithmic transformation of the data.

RESULTS

The results are presented in Table 1. All 15 cancers converted testosterone to 5α DHT, 5α androstanediol and Δ4 androstenedione but synthesis of oestradiol was detected in only 8 tumours. In certain tumours, these products represented only a small proportion of the total metabolites; uncharacterized highly-polar compounds being responsible for most of the unidentified material.

The addition of oestradiol-17β to the incubates caused significant inhibition of the metabolism of testosterone ($P<0.01$) and its

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Table 1. In vitro *effects of oestradiol* 17β *metabolism of* 7α^3H *testosterone by human breast carcinomas*

	Testosterone metabolised	5α DHT	Production of 5α Androstanediol	Δ4 Androstenedione	Oestradiol
	(ng)	(ng)	(ng)	(ng)	(ng)
1. Control	203.4	16.3	16.1	103.8	—
+OE2	102.3	12.9	9.1	7.8	—
2. Control	183.5	38.8	13.3	35.2	0.8
+OE2	107.5	17.4	7.5	23.1	0.6
3. Control	204.6	4.5	1.1	3.9	0
+OE2	120.0	3.7	1.0	2.0	0
4. Control	232.4	17.1	6.7	11.9	0.4
+OE2	209.2	8.9	2.1	7.8	0.4
5. Control	234.3	34.0	7.4	36.7	0.4
+OE2	258.8	20.6	2.4	3.7	0.2
6. Control	170.3	16.8	8.6	46.8	3.5
+OE2	188.5	12.0	7.5	33.9	2.0
7. Control	218.0	13.0	9.8	5.8	0
+OE2	213.4	6.4	3.1	4.2	0
8. Control	537.9	6.6	1.3	382.2	0.7
+OE2	365.7	7.0	1.3	188.3	0.8
9. Control	107.0	16.0	14.2	16.2	2.0
+OE2	69.4	13.9	15.3	8.4	1.7
10. Control	196.1	39.5	16.1	4.9	0
+OE2	214.8	29.6	9.6	2.4	0
11. Control	152.0	11.4	5.7	3.8	0
+OE2	129.0	9.7	2.7	4.0	0
12. Control	432.8	25.0	14.4	121.7	0.3
+OE2	381.0	17.8	19.1	95.3	0.3
13. Control	220.4	31.7	24.2	46.6	0
+OE2	152.4	29.8	14.3	18.0	0
14. Control	182.9	17.1	4.6	5.4	0.6
+OE2	214.9	8.8	2.1	3.9	0.5
15. Control	168.4	17.7	3.2	4.8	0
+OE2	68.0	30.6	7.1	2.6	0
Geometric control	211.8	17.4	7.3	19.8	
Mean +OE2	164.3	12.9	4.9	8.0	

conversion to 5α DHT ($P<0.01$), 5α androstanediol ($P<0.05$) and Δ4 androstenedione ($P<0.01$) as compared with control incubates. No effect was observed on the synthesis of oestradiol but the small conversions observed make comparisons difficult.

Oestradiol-3-methyl ether had no effect on the metabolism of testosterone in the two tumours (patients 1 and 8) in which its effect was investigated.

DISCUSSION

These results indicate that oestradiol-17β in general, inhibits the metabolism of testosterone by human breast cancer. However, there is considerable variation not only in its effect between tumours but also on the conversion of testosterone to individual metabolites in the same tumour. It is possible that differences in endogenous steroids within tumours may contribute to this variation. The concentration of oestradiol-17β added to the incubates was, in physiological terms, high. However, this was similar to that which has been used by others to study oestrogen sensitivity in human breast cancer [8] and pharmocological doses are used when treating patients with advanced breast cancer by oestrogen therapy.

That the effect of oestradiol-17β is not a non-specific effect of any steroid is shown by the fact that the same concentration of oestradiol-3-methyl ether was without effect in the two tumours to which it was added. Similar effects of oestrogen have, however, been described on 5α reduction of C19 steroids in the human adrenal cortex and prostate [9–10].

Although one of us reported that oestradiol-17β inhibited 5α reduction of testosterone in hormone dependent rat mammary tumours but not in two hormone independent tumours [5], there is no evidence for such a relationship in human tumours. If oestradiol's effect on steroid metabolism in human tumours were limited to those which are hormonally sensitive, one would not expect to observe

the general inhibitory effects described in this study. Further, in two patients with recurrent disease, endocrine treatment failed to induce remission despite demonstrable *in vitro* inhibition of 5α reduction by oestradiol-17β (patients 2 and 14).

Nevertheless, this study does indicate that metabolism of testosterone by human breast cancers is not an autonomous process but as in normal endocrine tissues, can be influenced by the addition of other hormones.

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Failure of Neuraminidase Treatment to Influence Tumorigenicity or Immunogenicity of Syngeneically Transplanted Rat Tumour Cells*

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Abstract—Vibrio cholerae *neuraminidase (VCN) and* Clostridium perfringens *neuraminidase (CPN) have been examined for their ability to reduce tumorigenicity of a range of seven different transplanted rat tumours. The enzymes released* 0.2 *to* 1.2 *μ mole sialic acid/*10^9 *cells, comparable to levels reported by other workers. However, in no case did treatment of tumour cells with either enzyme consistently reduce* in vivo *tumorigenicity over and above that caused only by the enzymes' buffer solution. Moreover, with a strongly immunogenic MCA-induced sarcoma, VCN treatment of cells did not improve their efficiency for active specific immunotherapy, and enzyme treatment of cells of a carcinogen induced mammary carcinoma, non-immunogenic by conventional criteria, did not render them immunogenic. Essentially, these studies fail to confirm the reduced tumorigenicity of neuraminidase treated cells, or their increased immunogenicity.*

INTRODUCTION

FOLLOWING the observations of Lindenmann and Klein [1] and Sanford [2] that tumour cells treated *in vitro* with neuraminidase showed reduced *in vivo* tumorigenicity, several other workers have demonstrated similar phenomena with a number of experimental tumours [3]. This has been interpreted as being due to modification of cell surface membranes following removal of sialic acid and/or permanent binding of enzyme molecules, facilitating host recognition and immune destruction. Moreover, the increased immunogenicity of enzyme treated cells has been reported to render them highly effective for active specific immunotherapy, particularly against transplanted 3-methylcholanthrene-induced sarcomas in the mouse [4], and this has led to their incorporation into vaccines for clinical immunotherapy [5, 6]. However, other groups have been unable to confirm the reduced tumorigenicity of neuraminidase treated cells [7, 8] and it has been ascribed, at least partly, only to direct cytotoxicity of the enzyme [9]. In addition, with some experimental tumours, neuraminidase treatment fails to augment their efficacy for immunotherapy [10, 11] and in some cases leads to positive facilitation of tumour growth [12].

In view of these conflicting reports the present studies were carried out to determine the influence of neuraminidase pretreatment on the tumorigenicity of cells of a range of transplanted rat tumours. In addition, the amount of sialic acid released has been quantitated, and the value of enzyme treated cells for active specific immunotherapy has been assessed.

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MATERIALS AND METHODS

Tumours

Tumours arose spontaneously or were induced with chemical carcinogens in rats of a Wistar derived, Nottingham Inbred strain (W/Not). Tumours were carried by routine subcutaneous transplantation in syngeneic rats of the same sex as the primary host. Sarcomas Mc7 and Mc40A were induced by subcutaneous implantation of 3-methylcholanthrene (MCA) [13]. Both are highly immunogenic, producing resistance to challenge in conventionally immunized animals against 5×10^6 tumour cells or whole grafts of tissue, although growing from inocula of 5×10^5 cells in untreated rats.

Hepatoma D23 was induced by feeding 4-dimethylaminoazobenzene. This tumour is moderately immunogenic, growing from an inoculum of 5×10^3 cells in untreated rats but producing resistance to challenge with up to 5×10^5 cells in immune animals [14, 15]. The ascitic variant of this tumour, D23As [16], used in some tests was similarly immunogenic, intraperitoneal injections of irradiated cells conferring protection against intraperitoneal challenge with up to 5×10^5 D23As cells.

Mammary carcinoma AAF57 was induced by repeated intraperitoneal administration of *N*-hydroxy-2-acetylaminofluorene. This tumour lacks detectable immunogenicity, pretreated rats failing to reject challenge with 1×10^3 cells, the minimum for growth in untreated control rats [17].

Epithelioma Sp1 arose spontaneously, and is only weakly immunogenic, immunized rats rejecting up to 5×10^4 cells compared with its minimum growth inoculum of 1×10^3 cells [18].

Fibrosarcomas Sp7 and Sp24 arose spontaneously and are weakly immunogenic, producing protection up to 1×10^5 and 1×10^3 cells respectively, the minimum inocula for growth [17].

Single cell suspensions of solid tumours were prepared by digestion of finely minced tissue in 0.25% (w/v) trypsin in Hanks' balanced salt solution (Baldwin and Barker, [14]) followed by washing and resuspension in medium 199. D23As cells were harvested by peritoneal aspiration with Hanks' solution, containing 5 units/ml heparin, washed and resuspended in medium 199.

The ability of tumour cells to exclude trypan blue was assessed by diluting suspensions in an equal volume of freshly prepared trypan blue dye (1 vol 4.15% w/v NaCl to 4 vol 0.2% w/v aqueous dye) followed by immediate microscopic examination.

Neuraminidase

Vibrio cholerae neuraminidase (protease free) was obtained from Behringwerke A.G. Marburg/Lahn, West Germany, as a sterile solution at 500 units/ml in pH 5.5 sodium acetate buffer.

Purified *Clostridium perfringens* neuraminidase (Type V) was obtained from Sigma Chemical Company, London as a freeze dried powder. It was dissolved in pH 5.5 sodium acetate buffer (0.05M sodium acetate/0.154M sodium chloride/5mM Ca^{2+}, pH adjusted with 1% v/v acetic acid) to 1000 units/ml, sterilized by passage through a 0.22 μM millipore filter and stored at $-20°C$.

The unit of enzyme used throughout is that defined by Mohr and Schramm [19] as the amount of enzyme to release 1 μg of *N*-acetyl-neuramininic acid from human α_1-acid glycoprotein, at pH 5.5 in 5mM Ca^{2+}, in 15 min at 37°C.

Release of sialic acid from viable tumour cells by neuraminidase

Single cell suspensions of tumour cells were sedimented by centrifugation at 30 g for 5 min, the supernatant removed and cells resuspended in 1–2 ml neuraminidase solution/1×10^8 to 2×10^8 cells. Control cells were incubated with pH 5.5 acetate buffer. Following incubation at 37°C for 30 min, the supernatant was removed, cells washed × 2 in 1 ml 0.15M NaCl and enzyme eluates and washings combined. The pooled eluates and washings were made 10% with respect to trichloroacetic acid (TCA) and the protein precipitate removed after 30 min by centrifugation at $1500 \times \boldsymbol{g}$. The clarified supernatants were stored at $-20°C$ for sialic acid assay. Cells were examined microscopically before and after treatment and their trypan blue exclusion compared with cells which had been retained in medium 199 at 37°C.

Assay of sialic acid

Sialic acid was assayed by a modification of the method of Warren [20]. The following reagents were used:—

(a) 0.2M sodium periodate in 9M orthophosphoric acid;

(b) 10% sodium arsenite in 0.5M sodium sulphate/0.1N sulphuric acid;

(c) 0.6% thiobarbituric acid in 0.5M sodium sulphate.

To 0.2–0.4 ml aliquots of test solution, in triplicate, was added 0.1 ml periodate solution, and samples incubated at room temperature for 20 min. One millilitre of arsenite solution was added, tubes incubated for a further 5 min and 3 ml thiobarbituric acid solution added. Samples were then heated in a boiling water bath for 15 min, and cooled in cold water for 5 min. The developed chromophore was extracted into an equal volume of cyclohexanone and optical density readings made at 549 nm with a light path of 1 or 2 cm against a blank of cyclohexanone extract of treated 10% TCA/0.15M sodium chloride.

The concentration of sialic acid was determined by reference to standard solutions of *N*-acetylneuraminic acid (at 0.025 to 0.4 μmole/ml) (crystalline synthetic *N*-acetylneuraminic acid, Type IV, Sigma Chemical Company, London). Standard solutions were prepared in 10% in 0.15M sodium chloride to be comparable with test samples of tumour cell eluates. The molecular extinction coefficient was found to be 59,000, comparable to that reported by Warren [20]. The amount of sialic acid released from tumour cells is expressed throughout as μmole/10^9 cells.

Total sialic acid content of tumour cells was measured by heating cells for 1 hr at 80°C in 0.1 N sulphuric acid. The cell debris was removed by centrifugation at $1500 \times g$ for 30 min and the assay carried out on the supernatant compared against standard *N*-acetylneuraminic acid solutions in 0.1 N sulphuric acid.

Assessment of tumorigenicity of neuraminidase treated cells

Tumour cells were suspended in neuraminidase (50–500 units/ml) in pH 5.5 acetate buffer at a concentration of 10^7–10^8 cells/ml, to give ratios of 5.25 units/10^6cells/ml. Control cells were suspended in pH 5.5 buffer alone, and a sample of stock cells retained in medium 199. Suspensions were incubated at 37°C for 30 min, washed ×2 in 10 ml medium 199, aliquots taken for trypan blue exclusion testing, and samples of stock, enzyme, and acetate treated cells diluted in medium 199 to a suitable concentration for injection. Cells of solid tumours were injected subcutaneously in the right flank. Rats were examined weekly for tumour growth, and mean tumour diameters calculated from caliper measurements in two planes. D23As cells were injected intraperitoneally and animals killed when moribund with ascitic growths.

Active specific immunotherapy of sarcoma Mc7

The efficiency of neuraminidase treated cells for active immunotherapy was assessed using the Mc7 active specific immunotherapy model previously described [21, 22]. Animals received a subcutaneous challenge inoculum of 5×10^5 to 2×10^6 Mc7 cells in one flank and a simultaneous treatment injection subcutaneously in the contralateral flank of 2×10^6 Mc7 cells (untreated, or previously exposed to 5 units/10^6 of neuraminidase) in admixture with 500 μg moist weight of BCG organisms (Glaxo Vaccine, B.P. (percutaneous) Glaxo Laboratories, Greenford, England). No progressive growth occurred from the mixed vaccine of Mc7 cells and BCG, and this led to concomitant control of challenge inocula in the majority of rats [21].

RESULTS

Vibrio cholerae *neuraminidase*

Quantitation of sialic acid release. Table 1 shows results of analyses of enzyme eluates from cells of six different transplanted rat tumours exposed to 5 units neuraminidase/10^6 tumour cells. With all tumours, detectable levels of sialic acid were present in enzyme eluates, at 0.21 to 1.00 μmole/10^9 treated cells. Sialic acid assays on acid digests of sarcoma Mc7 cells showed a total cell content of 3.6 μmole/10^9 cells. With only two tumours, sarcoma Mc7 and epithelioma Sp1, was sialic acid detectable in eluates of

Table 1. *Release of sialic acid from rat tumour cells by* Vibrio cholerae *neuraminidase*

Tumour	Untreated cells Trypan blue excluding (%)	Acetate-treated cells* Trypan blue excluding (%)	Acetate-treated cells* Sialic acid released (μmole/10^9 cells)	Enzyme-treated cells† Trypan blue excluding (%)	Enzyme-treated cells† Sialic acid released (μmole/10^9 cells)
Sarcoma Mc7	93	86	0.07	86	0.67
Hepatoma D23	96	77	N.D.‡	77	0.60
Ascitic hepatoma D23As	96	95	N.D.	94	1.00
Mammary carcinoma AAF57	92	90	N.D.	77	0.68
Sarcoma Sp24	92	77	N.D.	73	0.56
Epithelioma Sp1	90	62	0.06	58	0.21

*Cells treated with pH 5.5 acetate buffer alone.
†5 units/10^6 cells, 37°C, 30 min.
‡Not detectable, <0.01 μmole/10^9 cells.

cells exposed to only pH 5.5 acetate buffer. With most tumour cells examined, enzyme treatment reduced the proportion of cells capable of excluding trypan blue. This was most noticeable with hepatoma D23, mammary carcinoma AAF57 and the epithelioma Sp1, where only 58–77% of treated cells excluded the dye, whereas at least 90% of untreated cells were unstained. This toxicity could not be ascribed only to exposure to enzyme, however, since cells treated with only pH 5.5 buffer showed a similar, albeit smaller, increase in the proportion of cells taking up the dye.

Tumorigenicity of treated cells. Table 2 summarizes the results of tests to assess the *in vivo* tumorigenicity of neuraminidase treated tumour cells. Cells exposed to enzyme, at the ratio of 5–25 units/10^6 cells were, after washing in medium 199, injected subcutaneously into syngeneic recipients. Groups of control rats received cells treated with only pH 5.5 acetate buffer, and untreated stock cells. In each test care was taken

Table 2. Influence of Vibrio cholerae *neuraminidase treatment of tumorigenicity of rat tumour cells*

Tumour	Enzyme treatment (units/10^6 cells)	Cells excluding Trypan blue %	No. cells injected*	Tumour takes
Sarcoma Mc7	5	86	1×10^6	3/6
	0†	86	1×10^6	4/6
	–	93	1×10^6	3/5
	5	82	1×10^6	5/6
	0	95	1×10^6	5/6
	–	95	1×10^6	3/5
	25	57	1×10^6	4/4
	0	71	1×10^6	4/4
	–	95	1×10^6	4/4
Hepatoma D23	25	67	1×10^4	0/5
	0	74	1×10^4	5/5
	–	91	1×10^4	5/5
	25	82	1×10^4	4/5
	0	67	1×10^4	4/5
	–	98	1×10^4	5/5
	5	77	1×10^4	4/5
	0	77	1×10^4	5/6
	–	96	1×10^4	5/5
Mammary carcinoma AAF57	5	93	1×10^4	5/5
	0	94	1×10^4	3/5
	–	98	1×10^4	4/5
	5	93	5×10^3	4/4
	0	94	5×10^3	4/5
	–	98	5×10^3	5/5
	25	78	5×10^4	5/6
	0	82	5×10^4	6/6
	–	91	5×10^4	6/6
Sarcoma Sp7	5	78	5×10^5	4/4
	0	79	5×10^5	4/4
	–	77	5×10^5	4/4
Sarcoma Sp24	5	73	5×10^4	3/5
	0	77	5×10^4	3/5
	–	92	5×10^4	5/5
Epithelioma Sp1	5	82	2×10^4	6/6
	0	85	2×10^4	6/6
	–	87	2×10^4	6/6
	5	58	1×10^4	5/6
	0	62	1×10^4	5/6
	–	90	1×10^4	3/6
	5	58	5×10^3	4/6
	0	62	5×10^3	1/6
	–	90	5×10^3	3/6

*Number trypan blue excluding cells.
†Treated with pH 5.5 acetate buffer alone.

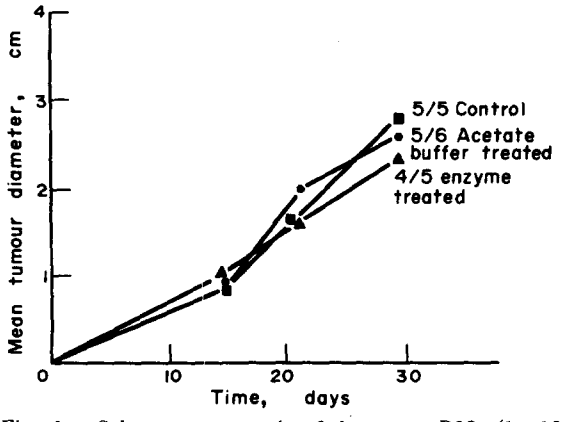

Fig. 1. *Subcutaneous growth of hepatoma* D23 (1×10^4 *cells*). *Cells were pretreated with* Vibrio cholerae *neuraminidase* (5 units/10^6 *cells*), *or* pH 5.5 *acetate buffer alone.*

to inject the defined number of trypan blue excluding cells, since in some cases enzyme treatment greatly increased the proportion of cells taking up the dye.

With none of the 6 tumours examined was there any consistent reduction in *in vivo* growth potential over and above that caused by only pH 5.5. acetate buffer (Table 2, Fig. 1). For example, with hepatoma D23, in the first test, 1×10^4 neuraminidase treated cells failed to grow in all of five rats, compared with growth in 5/5 animals in groups receiving acetate buffer treated or stock cells. This was not reproducable in two further tests, however, and is probably attributable only to direct toxicity of the enzyme for the cells as evidenced by reduction in the trypan blue excluding populations from 91 to 67%. The rats which had injected 1×10^4 enzyme treated D23 cells were challenged 30 days later with 5×10^3 untreated D23 cells subcutaneously in the opposite flank. Tumours developed in 4/4 animals, comparable to growth in new controls rats (4/4). With none of the other tumours was there a single test in which neuraminidase treatment prevented growth, and in some cases tumour incidence were higher following enzyme exposure than from untreated cells. Thus with epithelioma Sp1 an inoculum of 5×10^3 neuraminidase-treated cells grew out in 4/6 rats, compared with only 1/6 animals receiving cells exposed only to pH 5.5 buffer and 3/6 rats receiving cells from the untreated stock suspension.

A further series of tests was carried out with the ascitic variant of the hepatoma D23, D23As (Table 3). Cells were harvested from the peritoneal cavity, washed and treated with neuraminidase without the need for trypsin digestion. Again, however, enzyme treatment at up to 25 units/10^6 cells was totally without influence on the ability of the cells to form rapidly fatal ascitic growths, from inocula as low as 5×10^3 cells.

Table 3. *Influence of* Vibrio cholerae *neuraminidase treatment on ascitic growth of rat hepatoma D23As cells*

Enzyme treatment (units/10^6 cells)	Cells excluding trypan blue (%)	No. cells injected*	No. rats developing ascites	Survival (days)
5	86	1×10^6	5/5	5×13
0†	88	1×10^6	5/5	5×13
–	94	1×10^6	5/5	5×13
5	86	1×10^5	5/5	5×17
0	88	1×10^5	5/5	5×17
–	94	1×10^5	5/5	5×17
5	94	1×10^4	4/5	19, 22, 22, 22, 1 Rat>40
0	95	1×10^4	3/5	21, 21, 18 2 Rats>40
–	96	1×10^4	5/5	5×21
5	94	5×10^3	5/5	20, 20, 20, 21, 21
0	95	5×10^3	5/5	20, 20, 20, 20, 23
–	96	5×10^3	5/5	20, 20, 21, 21, 21
25	76	1×10^4	5/5	5×19
0	79	1×10^4	5/5	5×19
–	91	1×10^4	5/5	5×19

*Number of trypan blue excluding cells.
†Treated with pH 5.5 acetate buffer alone.

Table 4. Active specific immunotherapy of sarcoma MC7 with Vibrio cholerae *neuraminidase treated tumour cells and BCG*

Expt.	Challenge inoculum (No. cells)	Tumour growth in rats treated with vaccine* of:— VCN† treated MC7 cells + BCG	Acetate-treated‡ MC7 cells + BCG	MC7 cells + BCG	Controls
1	5×10^5	2/5	1/5	1/5	4/5
2	5×10^5	0/5	1/5	3/5	5/5
3	5×10^5	3/5	1/5	1/5	5/6
4	5×10^5	3/6	2/6	2/6	3/3
	Totals =	8/21 (38%)	5/21 (24%)	7/21 (34%)	17/19 (90%)
5	1×10^6	3/5	2/5	—	5/5
6	2×10^6	4/5	5/5	4/6	—

*2×10^6 Mc7 cells admixed with 100 to 500 μg moist weight BCG organisms injected contralaterally to challenge inoculum. No tumours developed from these mixed inocula.
†5 units/10^6 cells.
‡Treated with pH 5.5 acetate buffer alone.

Active specific immunotherapy with enzyme treated cells. To examine neuraminidase treated cells for increased immunotherapeutic efficiency a series of tests was carried out with the sarcoma Mc7 (Table 4). Animals received challenge inocula of 5×10^5–2×10^6 cells and were treated at a contralateral subcutaneous site with a vaccine of 2×10^6 viable Mc7 cells mixed with 500 μg BCG. Cells in this vaccine were treated with neuraminidase, or acetate buffer, and their therapeutic effectiveness compared with untreated cells admixed with BCG. It has previously been established that mixed inocula of Mc7 cells and BCG fail to produce progressively growing tumours and that there is concomitant generation of systemic tumour-specific immunity capable of suppressing growth of the contralateral challenge inocula in a high proportion of rats [21, 22]. In the first series of the present tests, with challenge inocula of 5×10^5 Mc7 cells (Expts. 1–4, Table 4), there was no evidence that neuraminidase treated cells gave a better therapeutic response than control cells. For example, in experiment 2, cells + BCG controlled tumour growth in only 2/5 rats, compared with suppression in all animals receiving enzyme treated cells and BCG, but in the third test these responses were reversed. The overall tumour incidences in the first four experiments were comparable in animals receiving enzyme treated cells and untreated cells (38% incidence compared with 34%), while the best response was given by cells exposed to only pH 5.5 acetate buffer (24% tumour incidence).

In the fifth and sixth tests the challenge inocula were increased to 1×10^6 and 2×10^6 cells respectively, at and above the limit normally controllable by a standard treatment inoculum of 2×10^6 Mc7 cells + BCG [21]. In neither test, however, were enzyme treated cells better than acetate treated cells or untreated cells, tumour incidence being comparable in all groups.

Immunisation against mammary carcinoma AAF57 with neuraminidase treated cells. Mammary carcinoma AAF57 lacks demonstrable immunogenicity, so that surgical removal of growing subcutaneous grafts or repeated implantation of irradiated cells fails to protect against a viable challenge inoculum of $1 \times 10^3 - 2 \times 10^3$ cells, the minimum for consistent growth in control rats [17]. In the experiment shown in Table 5 the immunizing capacity of AAF57 cells treated with neuraminidase was examined. Rats received three subcutaneous injections of 1×10^7 neuraminidase treated, irradiated AAF57 cells and were subsequently challenged with 2×10^3 untreated AAF57 cells. Tumours developed in 5/6 rats, compared with 7/7 takes in animals receiving irradiated acetate buffer exposed or untreated cells, and control rats.

Clostridium perfringens *neuraminidase*

In view of the failure of *Vibrio cholerae* neuraminidase to reduce tumorigenicity of the transplanted rat tumours examined, a further series of tests was carried out with an alternative preparation of the enzyme, that from *Clostridium perfringens*. The results were, however, comparable to those with the *Vibrio* preparation. Thus incubation in the enzyme at 5 units/10^6 cells released

Table 5. *Immunization of rats with* Vibrio cholerae *neuraminidase treated mammary carcinoma AAF57 cells*

Immunizing inocula		Challenge inocula		
Cells treated with:	Injections	No. cells	Tumour takes	
			Test	Control
Enzyme*	$3 \times (1 \times 10^7$ Irradiated cells†)	2×10^3	5/6	
Acetate	$3 \times (1 \times 10^7$ Irradiated cells†)	2×10^3	7/7	7/7
—	$3 \times (1 \times 10^7$ Irradiated cells†)	2×10^3	7/7	

*5 units/10^6 cells.
†15,000 ^{60}Co γ-irradiation after enzyme treatment.

Table 6. *Release of sialic acid from rat tumour cells by* Clostridium perfringens *neuraminidase*

Tumour	Untreated cells Trypan blue exclusion (%)	Acetate-treated cells* Trypan blue exclusion (%)	Acetate-treated cells* Sialic acid released (μmole/10^9 cells)	Enzyme-treated cells† Trypan blue exclusion (%)	Enzyme-treated cells† Sialic acid released (μmole/10^9 cells)
Sarcoma Mc40A	96	79	0.20	74	1.28
Mammary carcinoma AAF57	94	86	N.D.‡	92	0.48
Sarcoma Sp24	88	77	0.05	72	0.51

*Cells treated with pH 5.5 acetate buffer alone.
†5 units/10^6 cells, 37°C, 30 min.
‡Not detectable, <0.01 μmole/10^9 cells.

Table 7. *Influence of* Clostridium perfringens *neuraminidase treatment on tumorigenicity of rat tumour cells*

Tumour	Enzyme treatment (units/10^6 cells)	Cells excluding trypan blue (%)	No. cells injected*	Tumour takes
Sarcoma Mc40A	5	85	1×10^6	5/5
	20	85	1×10^6	5/5
	0†	79	1×10^6	5/5
	–	98	1×10^6	5/5
Mammary carcinoma AAF57	5	85	1×10^5	3/4
	20	66	1×10^5	4/4
	0	90	1×10^5	4/4
	–	94	1×10^5	4/4
Hepatoma D23	1	71	1×10^4	5/5
	0	77	1×10^4	2/5
	–	83	1×10^4	2/4
Sarcoma Sp24	5	72	5×10^4	3/4
	0	71	5×10^4	4/4
	–	88	5×10^4	2/4

*Trypan blue excluding cells.
†Treated with pH 5.5 acetate buffer alone.

0.48–1.28 μmole sialic acid/10^9 cells from the sarcoma Mc40A, mammary carcinoma AAF57 and sarcoma Sp24 (Table 6). Enzyme treated cells showed, however, no reduced *in vivo* growth potential, tumour incidence from low cell inocula being at least as high as that from cells treated with only pH 5.5 acetate buffer (Table 7).

DISCUSSION

Treatment of tumour cells with neuraminidase has been reported to reduce their

tumorigenicity with several different cell types including adenocarcinomas [2], leukaemias [23–25], carcinogen-induced fibrosarcomas [26–29] and ascitic tumours [30, 31]. *Vibrio cholerae* neuraminidase has been reported to be more effective than the enzyme from *Clostridium perfringens* [30]. In addition to reduced growth potential, enzyme treated cells show increased immunizing potential, and active therapy with them is highly effective against MCA-induced sarcomas in the mouse, including postsurgical residual disease [4, 32]. Based on these observations with experimental tumours Sedlacek *et al.* [33] have reported beneficial effects in immunotherapy of spontaneous mammary tumours in the dog with neuraminidase treated cells and clinically neuraminidase treated cells have been used for treatment of patients with melanoma [34], leukemia [5], and carcinoma of the breast, gastro-intestinal tract and ovary [6].

Decreased tumorigenicity, and enhanced immunogenicity of experimental tumours following neuraminidase treatment is thought to reflect enzyme-induced changes at the cell surface. Thus *Vibrio cholerae* neuraminidase (VCN) splits 2–3, 2–6 and 2–8 glycosidic linkages between terminal *N*-acylated neuraminic acids and cell surface sugar residues, while *Clostridium perfringens* neuraminidase (CPN) cleaves predominantly the 2–3 linkages [3, 9]. In addition neuraminidase may bind to cell surfaces, remaining attached and enzymatically active even after excessive washing [35, 36]. These modifications of the cell surface may reduce electro-negative charge, increase cell deformability and facilitate host recognition [3, 9].

In contrast to the reports of benificial effects of neuraminidase treatment, the present studies, using a range of seven different tumours, showed no consistent reduction in the tumorigenicity of VCN or CPN treated cells, over and above that ascribable to non-specific damage, although levels of sialic acid removed from the cells were comparable to those reported by other workers (e.g. 0.25 μmole/10^9 MCA-induced sarcoma cells in the mouse [28], 0.72 μmole/10^9 L1210 leukaemia cells in the mouse [23]. The tumours employed covered a range of immunogenicities, and were selected to optimise the possibility of detecting reduced tumorigenicity as a result of increased immunogenicity. The immunological nature of reduced tumorigenicity of neuraminidase treated cells in other systems is demonstrated by its abrogation in immuno-suppressed hosts [26, 27, 29]. In the present study animals which had rejected enzyme treated cells of the immunogenic hepatoma D23 were not immune to a further challenge with D23 cells, implying that in this case the rejection of the initial inoculum was not immunologically mediated.

Currie and Bagshawe [30] found that exposure of neuraminidase treated cells to trypsin reversed the beneficial effect of VCN, even after a subsequent, second, VCN incubation. In the present studies therefore tests carried out with the ascitic variant of the hepatoma D23, where trypsin exposure was not needed to prepare single cell suspensions for neuraminidase treatment. Again, however, neuraminidase treatment had no influence on their *in vivo* growth potential.

In addition to demonstrating no influence on tumorigenicity, the present studies also fail to demonstrate increased effectiveness of VCN treated cells of sarcoma Mc7 for active specific immunotherapy, sharply contrasting with the reports of Rios and Simmons [4] with MCA-induced sarcomas in the mouse, where established tumours may undergo specific immunological regression following active therapy with VCN treated cells. With the carcinogen induced mammary carcinomas AAF57, non-immunogenic by conventional criteria, neuraminidase treated cells, prevented from growth by irradiation, afforded no protection to viable tumour cell challenge, comparable to untreated irradiated cells.

In keeping with the present finding, however, other workers too have failed to demonstrate consistent beneficial effects following neuraminidase treatment of transplanted tumour cells. Thus Hauschka *et al.* [8] were unable to confirm the findings of Sandford [2] of reduced tumorigenicity of TA3 mouse mammary carcinoma cells, other than that attributable only to direct cytotoxicity of the enzyme. Similarly Yuhas *et al.* [7] working with a transplanted lung carcinoma in the mouse found no reduced tumorigenicity of VCN-treated cells, and demonstrated toxicity of the enzyme by *in vitro* studies, including ^{3}H-thymidine incoporation. With the B16 mouse melanoma Froese *et al.* [12] found no reduced tumorigenicity following VCN treatment, and enhanced tumour growth following pre-immunization with VNC-treated, irradiated cells. In a more recent series of studies with the Landeschütz ascites tumour trans-

planted into three strains of mice Smyth *et al.* [31] observed no reduced tumorigenicity of VCN treated cells in A2G mice, contrary to the earlier finding of Currie and Bagshawe [30] with the same tumour line and mouse strain. In other mouse strains (Schofield and BDF/1) reduced growth potential was only detectable with inocula below the LD_{100} in control animals. Similarly there was no improved beneficial effect of VCN treated cells for immunotherapy of an intracranially transplanted mouse glioma [10], while VCN treated irradiated cells were less efficient for immunotherapy of a mouse leukaemia than simply irradiated cells [11].

Essentially these studies indicate that, while neuraminidase may be efficient in many other experimental systems, it fails to influence the growth or immunotherapeutic effectiveness of transplanted rat tumour cells. This observation, together with other similar reports, including enhancement of growth, emphasises the need for caution in assuming the enzyme to be generally effective, especially in the clinical situation.

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Intravenous BCG Therapy of Mammary Carcinoma in Bitches After Surgical Excision of the Primary Tumour

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Abstract—*Thirty four bitches with defined histological forms of mammary carcinoma were studied post-mastectomy for over* 100 weeks. *They were randomized into* 3 *groups: (a) no further treatment* (11), *(b) placebo treatment* (10), *(c) intravenous BCG therapy* (13). *The mean survival time of the control groups was* 24 weeks *whereas for the treated group* 100 weeks. *This was significantly different* ($P<0.05$) *as calculated by the Chi² test* (χ^2).

INTRODUCTION

As IN women [1, 2] the mammary glands of bitches are the commonest single site for the development of neoplasms. Over 50% of all tumours in the female dog arise in the mammary tissues [3], half of the total number being histologically benign [4]. The overwhelming majority of the remainder can be classified morphologically as carcinomas. Canine mammary carcinomas normally exhibit clinical signs of malignancy by metastasizing to the regional lymph nodes and lungs, but unlike similar neoplasms in women very rarely spread to the bones, CNS or adrenal glands [4]. Inoperable local recurrence frequently complicates the clinical syndrome but is unusual in the absence of metastatic spread.

Retrospective follow-up studies on bitches treated surgically for mammary neoplasia [5] have shown that 60–70% of animals with an invasive mammary carcinoma die as a result of the initial tumour within a year of surgery, in spite of having no evidence of metastases when surgery was undertaken. The existence of this easily identified group of bitches, which can readily be predicted to develop metastases, closely resembles the situation in women with evidence of lymph nodal spread at mastectomy [6]. Any method of delaying the onset of metastatic disease is therefore of great comparative interest.

Owen and Bostock [7] demonstrated that pulmonary irradiation could increase the number of pulmonary metastases in certain cases. One possible mechanism involved in the observed increase may have been the destruction of some local immune mechanism. These workers subsequently reported that a course of intravenous BCG following complete excision of primary osteosarcoma delayed the development of pulmonary metastases [7, 8]. However, because of the extremely poor, and well documented, prognosis following amputation of a limb in canine osteosarcoma [9], it was difficult to justify the inception of a randomised controlled trial. These limitations do not apply to mammary carcinoma, where surgical excision of the primary is the treatment of choice and the effect of subsequent intravenous BCG on survival time can be justifiably compared to a control group receiving surgery alone.

MATERIALS AND METHODS

Spontaneously arising mammary neoplasms were surgically excised and submitted to the Department of Clinical Veterinary Medicine, Cambridge University for histological diagnosis. Tumours were classified

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according to the criteria which have been previously described [5] into fibroadenomas, carcinomas and sarcomas. The carcinomas were further subdivided into either well defined or invasive on the basis of the boundary between the tumour and surrounding connective tissues. All dogs with an invasive carcinoma, either of solid or tubular morphology, were then subjected to a thorough clinical examination three weeks after surgery. Bitches with no palpable local or lymph node metastases and no radiological evidence of pulmonary metastases were randomised into three groups: (1) surgery alone, (2) surgery alone plus placebo, (3) surgery plus intravenous BCG.

The treated group received the contents of one vial of lyophilized BCG (Glaxo percutaneous strength) suspended in 5 ml of normal saline at a dose of 0.1 mg BCG/kg body weight. Prior to each injection each animal was injected intramuscularly with chlorphenirmine maleate BP[1] to avoid anaphylactic-like reactions following injection. This procedure was repeated at intervals of 1, 2 and 4 weeks and then every 8 weeks until 1 yr after surgery, when all treatment was ended. One control group received placebo treatment of intramuscular Piriton* and 5 ml of intravenous saline and the other had no further treatment. The study was continued for at least 100 weeks after surgery or until death, the cause being determined by post-mortem examination.

The survival time of dogs in the treated and control groups were compared using the Wilcoxon rank sum test and death rates with the Chi[2] test. In the former test, animals still alive were treated as equivalent at the highest rank.

*"Piriton", Allen and Hanburys Limited, London England.

RESULTS

Intravenous BCG was well tolerated in most cases, with only a transient pyrexia, malaise, and inappetence for 48–96 hours after each injection. Some dogs appeared to show a more prolonged depression after the fourth and fifth injection. However, only one dog had to have treatment withdrawn due to continued anaphylactic attacks despite premedication with Piriton (this case not included in results).

A total of 34 cases of histologically confirmed invasive mammary carcinoma were seen at this department three weeks post mastectomy. The bitches were assessed as tumour-free by the criteria described in Materials and Methods and randomised into three groups. The median survival time for each group is shown in Table 1. As can be seen this was very similar in the two control groups. However, the BCG treated group was significantly different from these controls ($P<0.02$). The median survival time could only be estimated (at 100 weeks) compared to 24 weeks for the controls, since the median bitches were still alive at the time of writing. The death rate in the first post-surgical year was significantly greater in the control groups (50 and 56%) than in the treated groups (15%) where only two dogs died as a direct result of the tumour. Where death is referred to in the text or tables this usually represents euthanasia in the terminal stages of illness, although four dogs in this study died of natural causes.

In Table 2 the findings in all those animals destroyed because of the tumour at the end of the follow-up period are

Table 1. *The effect of intravenous BCG on the median survival time and death rate of bitches following excision of an invasive mammary carcinoma*

Group	Number of dogs	Mean age at surgery (yr)	Median survival time (weeks)	%Dead after 1 yr	
				All causes	Tumour
Control surgery only	11	8.5	24	64	56
Control surgery + placebo	10	9.5	24	70	50
Treated surgery + BCG	13	10.0	100 (estimated)	23	15

Table 2. The cause of death in dogs following surgical excision of an invasive mammary carcinoma

Group	% Dead at time of writing	Number of bitches with: Local recurrence	Lung metastasis	Recurrence and metastasis	Other causes
Controls	81	1*	8	4	4
Treated	54		1	2	4

*No post-mortem was allowed on this animal.

summarised. As can be seen, 17 of the 21 control bitches were dead, 13 as a result of the original tumour and 4 of other causes. In one of the control animals still alive local recurrence and pulmonary metastases have been radiographically confirmed. In contrast 3 of the 13 BCG treated dogs died from the mammary carcinoma, whilst 4 had succumbed to unrelated diseases. Local recurrence was present in 24% of the controls and 15% of treated dogs whilst 57% of controls compared to 23% of treated dogs developed pulmonary metastases.

Survival curves for the combined control groups, and treated group, are compared in Fig. 1. The difference in overall survival rates is significant up to about 1 yr. After this, the rates become almost identical as deaths from other causes begin to exert their influence.

DISCUSSION

The survival curve for control bitches with invasive carcinoma in this study was very similar to that previously reported [5]. It illustrated the high mortality associated with recurrent or metastatic tumour during the first post-surgical year, followed by a lower rate due to other diseases. In the treated animals this initial high mortality was almost completely abolished and the death rate was linear over the whole follow-up period. There was no evidence that the incidence of local recurrence was reduced by intravenous BCG therapy and the results indicated that the main effect was a delay in the onset of pulmonary metastases. This was similar to the findings with canine osteosarcoma [7, 8].

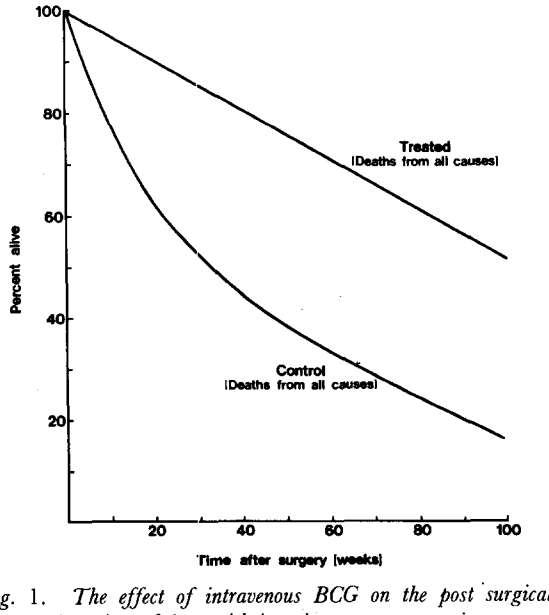

Fig. 1. The effect of intravenous BCG on the post surgical survival time of dogs with invasive mammary carcinoma.

Intravenous BCG in dogs was shown to induce a diffuse macrophage infiltration into the lung with focal granuloma formation [7, 10] and characteristic epithelioid granulomas were also identified in the liver. This resembled the reports of granuloma formation in man following intravenous and intralesional BCG administration [11–15].

The mechanism of the delay in onset of pulmonary metastases has been investigated in this unit. Lymphocytotoxicity studies have failed to provide conclusive evidence that non-specifically activated non-adherent cells were present following intravenous BCG [16]. However experiments have shown that mononuclear phagocytes associated with the pulmonary granulomas exhibit a marked non-specific tumour cell lysis *in vitro* [10]. This was not found with mononuclear phagocytes isolated from normal canine lungs.

The evidence suggested that the delay in onset of metastases *in vivo* was mediated by a non-specific mononuclear phagocyte response. This concept would support the experimental work of Baldwin and Pimm [17–19] who have demonstrated that intravenous BCG controlled pulmonary metastases without affecting the primary tumour. This was suggested to be comparable to the

restriction of tumour growth when BCG is administered locally [20–23].

The observation that intravenous BCG will significantly prolong the post-surgical survival time of dogs with mammary carcinoma is of great comparative interest. However, reports of the efficacy of intravenous BCG in man are conflicting and may obviate a direct extension from this useful canine model of spontaneous neoplastic disease. Further work in the dog should be aimed at identifying fractions of the organism which are responsible for the observed clinical improvement and improving the therapy regime [10]. This will require the study of a large number of animals and can only be done with the co-operation of a number of workers in different centres.

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Multivariate Biochemical Indicators of Breast Cancer: An Evaluation of Their Potential in Routine Practice*

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Abstract—*A vertical study has been made of the frequency of abnormalities of the levels of* 3 *tumour related antigens (CEA, βHCG and casein),* 2 *serum enzymes (gamma glutamyltranspeptidase and alkaline phosphatase),* 4 *acute phase reactant proteins (haptoglobin, acid glycoprotein, ceruloplasmin and antitrypsin) and a random study of C-Reactive protein in* 429 *women attending a breast clinic.*

Whilst the frequency of abnormality increases with tumour load, these parameters seem unable to discriminate high risk groups Stages II *and* III *from benign breast disease. The women are heterogenous with respect to their individual marker profiles. Elements within this panel of potential markers could be used to design a profile for longitudinal monitoring of recurrence in certain high risk groups, but as yet they do not appear to warrant their introduction into routine medical practice until they have been proven to aid clinical decision making.*

INTRODUCTION

THERE is still much controversy as to the optimum plan of therapy of breast cancer in women, be they pre- or post-menopausal. All will agree that the eventual outcome depends on whether the tumour has produced micro-metastases or overt metastases at the time of first presentation and whether local treatments can effectively prevent any residual local focus continuing as a source of metastases. The natural history of the disease is such that the evaluation of the combination of various modalities of treatment usually takes between 5 and 8 yr to provide an answer and even then it says nothing about the really late recurrences. Undoubtedly a reliable system of tumour markers to provide evidence of micro-metastases or an early warning of growth in previous dormant micro-metastases would be of help in planning the treatment for the individual patient; in particular the necessity for, and the timing of adjuvant chemotherapeutic or hormonic therapy.

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Several tumour related antigens (TRA) have been studied in breast cancer, carcinoembryonic antigen [1–3], β sub-unit of human chorionic gonadotrophin [4, 5] and kappa casein [6, 7]. In the main, these have been looked at as single tests. The overall impression is that these products can only be found in a proportion of women with breast cancer and this proportion increases slightly with the progression of the disease. Franchimont and his colleagues [8] have advocated that coincidental measurement of 4 tumour related antigens will result in the finding of at least one positive value in 69% of patients with T1–3, N0, N1, M0 cancers and 88% in the presence of metastases. This multivariate approach has been extended by Coombes *et al.* [9] to the point where they have attempted to apply most known cancer tests, 19 in all, to a group of 51 patients, 42 having active breast cancer and 9 with benign breast disease. But such a large number of variates inevitably will result in a higher background of random positivity of one or more above the 95 percentile of whatever set of values are chosen as being representative of the non-cancer patients. In this study we have

investigated whether the multivariate approach could be the basis of a monitoring system and to test it by a limited battery of tumour related antigens, serum enzymes changes associated with metastatic cancer and a profile for acute phase reactant proteins (APRPs). These tests have been applied, in the main, to a population of women attending the breast clinic of a large general hospital.

MATERIALS AND METHODS

Patients

Four hundred and twenty nine of the patients attending the Combined Breast Clinic of the General Infirmary at Leeds, between October 1975 and February 1977, were the main subject of this study. They were divided into the following principal groups: 42 controls being women in whom no abnormality was detected (mean age 37.5 yr, range 19–68); 100 with benign breast disease (mean age 44.1 yr, range 18–74); 48 with primary cancers (mean age 56.5 yr, range 29–82); and 238 patients attending the clinic after diagnosis and treatment of primary breast cancer. The latter group was divided into: 156 post-mastectomy clinically tumour free (mean age 59.0 yr, range 26–82); 52 with metastases (mean age 58.0 yr, range 36–76) 20 of whom were receiving medical treatment; and 30 women (mean age 70.0 yr, range 49–91) receiving medical and X-ray treatment for primary tumours, 11 of them having evidence of distant metastases. In addition, 133 sera from women (age not known) with primary breast cancer were received, 84 being from the Ludwig Institute, Lausanne and 49 from the Royal Marsden Hospital, London, for protein profile determinations only. The statistical study of the incidence of positive markers was based on a single blood sample. In the primary tumour group the blood was drawn prior to any form of therapy. In the remaining groups, samples were taken at least 6 weeks after surgery or X-ray therapy. In the patients with benign disease the samples were usually taken prior to biopsy. In all cases the diagnosis was based on appropriate histological examination. A limited number of sequential samples taken over a period of one year have been examined in some patients to investigate the stability of the markers within a given individual. During the course of the investigation 6 patients in the Leeds series were reported to have died of metastatic cancer; samples of all these women were taken at a time when they were still fit enough to attend the clinic. None of the patients in this series was sufficiently incapacitated by their disease to be considered in a terminal phase at the time of examination.

Methods

Carcinoembryonic antigen (CEA). CEA was assayed in serum (100 μl) by double antibody radioimmunoassay [10] using goat anti-CEA (Ace 36) kindly donated by Professor C. W. Todd, and horse antigoat antiserum (BW 402) from Burroughs Wellcome, the assay being adapted for automation [11].

β sub-unit of human chorionic gonadotrophin (βHCG). βHCG was measured according to the method of Kardana and Bagshawe [12].

K-Casein. K-Casein was assayed by an automated double antibody assay [11] performed in serum (100 μl) as described by Woods *et al.* [7].

The detection limit is 10 μl/l K-Casein; the within assay coefficient of variation was 3%, inter-assay coefficient of variation 18%.

Acute phase reactant proteins. α_1 Acid glycoprotein (AGP), α_1 antitrypsin (AT), ceruloplasmin (Cp), C-Reactive protein (CRP) and haptoglobin (Hp) were determined by single radial immunodiffusion [13], using antisera, standards and standard control plasma supplied by Behringwerke AG, Marburg, Lahn, Germany. The standards and standard control plasma were applied to each plate. The values for haptoglobin were corrected for differences in the molecular size of the various phenotypes. The correction factors were Hp1:1 $\times$ 0.6, Hp2:1 $\times$ 1.3, Hp2:2 $\times$ 1.5. The phenotypes were identified by electrophoresis in gradient polyacrylamide gels according to Baxter and Rees [14]. The choice of the proteins to be included in the profile was based on experience of other solid tumours [15, 16] and previous reports on Cp in breast cancer [17]. The day to day coefficient of variation (S.D./Mean $\times$ 100) of repeated assays over the whole range of values was AGP 6.7%, AT 9.4% and Hp 11.3%, the inaccuracies being greatest at the upper and lower limits of the assays.

Serum enzymes. Gamma glutamyltranspeptidase (GGT) (EC 2.3.2.1) was measured by the method of Jacobs [18] with the assays run

at 37°C. Alkaline phosphatase (ALKP) (EC 3.1.3.1) was determined by the *p*-nitrophenylphosphate method of Bessey, Lowry and Brock [19]. The enzyme assays were standardized using pooled serum from normal donors.

Statistical analysis. The 95th percentile of the values of the various proteins and enzymes in the controls and women with benign breast disease were determined by probit analysis with logarithmic transformation. Levels above these values were used as discriminants for reporting "positivity" in certain of the tables. Analysis of variance and Student's *t*-test were all made on log transformed data to compensate for the skewness in some of the distributions.

All the statistical analyses were run on the University of Leeds ICL 1906A computer using programs from the Statistical Package for Social Sciences.

RESULTS

Tumour related antigens

The frequency of elevation of CEA, βHCG and casein in the various groups of patients is shown in Table 1. It can be seen that whilst there is a higher incidence of raised levels of tumour related products in the blood of patients with metastatic cancer, both prior to treatment and whilst receiving treatment, these markers were low in incidence in Stages I–III cancer and could not be used as the basis of differential diagnosis from benign diseases of the breast. Likewise on the basis of a single reading in patients following mastectomy and thought to be clinically free of tumour, there is sufficient overlap with the benign disease to preclude using them as the basis of firm diagnosis of metastatic cancer, although a rising titre is extremely suspicious. In the patients who were being followed up routinely following surgery or medical treatment for a primary tumour, only 5/22 of the patients with an elevated CEA and 1/24 with elevated casein, showed consistent elevations of these markers on consecutive measurements and none of the 21 patients with an elevated βHCG showed persistent elevation of this marker.

Acute phase reactant proteins and enzymes

Table 2 shows the means and standard deviations of the 2 enzymes investigated and of the 4 APRPs. Patients receiving medical treatment, which is mainly hormonal manipulation, have been excluded as the pharmacological effects of this treatment have a direct effect on the synthesis of several of the markers [20]. An analysis of variances showed that significant differences occurred between and within the groups for Hp, AGP and Cp but the variation of AT in the different patient populations was not highly significant.

Further analysis showed that the levels of Hp in the primary tumours of stages I–III were significantly different ($P = < 0.001$) from that of stage IV and the clinically free patients on follow-up were significantly different at the same level from those with proven untreated metastatic cancer. The noticeable feature of the behaviour of the serum enzymes is the very large variances encountered in metastatic cancer; this is a reflection of particularly high values in some of the patients with clear-cut evidence for hepatic metastases as indicated by raised GGT and alkaline phosphatase or those with a high alkaline phosphatase and normal GGT as observed when there is an established metastasis in bone.

Table 3 shows the frequency of values

Table 1. *Incidence of Leeds patients with raised levels of tumour related antigens*

	CEA >5 μg/l	βHCG >5 m i.u./ml	Casein >60 μg/l	Number of positive patients with more than one marker raised
Control	0/32	2/30	0/30	0
Benign	2/79	6/71	7/94	3
Stages I, II and III	1/40	1/30	5/41	0
Post-mastectomy Clinically tumour free	7/142	12/122	12/148	6
Stage IV and untreated metastatic cancer	9/31	6/27	4/34	4
Metastatic cancer receiving treatment	7/46	10/47	10/38	6

Table 2. Means, standard deviations, analysis of variance and t-tests for the acute phase reactant proteins and enzymes estimated on Leeds, Royal Marsden Hospital and Swiss patients

Group	Group No.	*n*	Hp g/l	α_1 AGP g/l	Cp g/l	α_1 AT g/l	Alk P i.u./l	GGT i.u./l
Control	1	42	2.8±1.5	0.7±0.4	0.34±0.10	3.1±1.3	19.6 ± 7.8	14.7 ± 7.7
Benign	2	100	2.6±1.3	0.8±0.4	0.32±0.13	2.9±1.0	22.4 ± 7.8	14.2 ± 8.9
Stages* I, II and III	3	129	3.2±1.5	1.0±0.4	0.39±0.11	2.8±1.1	28.8†± 8.8	16.5†±11.6
Stage IV*	4	42	4.8±2.4	1.4±0.8	0.48±0.15	3.3±0.9	‡	‡
Post mastectomy Clinically tumour free	5	156	3.0±1.5	1.0±0.5	0.35±0.07	3.1±1.2	30.2 ±12.6	23.5 ±26.3
Post mastectomy untreated metastases	6	32	4.9±2.8	1.3±0.9	0.42±0.16	3.7±1.4	49.1 ±26.2	32.9 ±47.2
Analysis of variance§		501	$F\frac{5}{495}=10.420$	$F\frac{5}{494}=12.438$	$F\frac{5}{483}=13.619$	$F\frac{5}{490}=4.456$	$F\frac{5}{359}=25.777$	$F\frac{5}{361}=7.232$
Student's *t*-test§ $P=<0.001$ between groups			1 and 4 2 and 4 3 and 4, 3 and 6 5 and 4, 5 and 6	1 and 3, 1 and 4 2 and 3, 2 and 4 2 and 6 3 and 4 4 and 5	1 and 4 2 and 3, 2 and 4 3 and 4 4 and 5	3 and 6	1 and 3, 1 and 5 1 and 6 2 and 3, 2 and 5 2 and 6 3 and 6 5 and 6	2 and 5, 2 and 6

*At first presentation.
†Data in Leeds patients only.
‡Data available on only 2 patients from Leeds. Eliminated from analysis.
§Variables have been transformed logarithmically.

Table 3. Incidence of Leeds patients with raised levels of tumour associated products and of those with raised levels of tumour associated and/or tumour related products

	Hapt >5.2 g/l	AGP>1.4 g/l	Cpl >0.55 g/l	Alk Phos >33 i.u./l	More than one TA* product raised	Any one TA or TR marker raised‡	Any 2 TA or TR† markers raised‡	Any 2 TA and 1 TR or any 2TR and one TA markers raised‡
Control	3/42	3/42	2/41	2/41	2/41	26.2%	4.8%	0
Benign	6/100	5/100	5/98	4/99	2/99	29.0%	5.0%	0
Stages I, II and III Post mastectomy Clinically Tumour free	3/46	1/46	1/44	13/44	2/45	39.1%	8.6%	0
Stage IV and untreated metastatic cancer	14/156	19/156	1/150	44/155	14/155	48.1%	16.0%	5.1%
Metastatic cancer receiving treatment	14/34	11/34	5/34	23/32	16/32	93.8%	73.5%	20.6%
	6/49	11/50	9/41	23/46	14/46	82.0%	44.0%	14.0%

*Tumour associated.
†Tumour related.
‡Expressed as the minimum percentage.
Some patients excluded as all the markers were not estimated.

falling above the calculated 95th percentile (as determined for the benign group) for Hp, AGP, Cp and alkaline phosphatase. These figures have then been added to those for the tumour related antigens to show the relative frequency of various combinations.

By application of the binomial theorem it can be shown that by chance alone with a linear combination of 4 markers using the discriminant value of the 95th percentile of the benign breast disease group, the intrinsic probability of finding 1, 2, 3 or 4 markers positive is: 0.17, 0.014, 0.004 and 0.000006 respectively, that is assuming some of the women with cancer may also have coincidental benign disease. For this reason, we have used two of the non-specific markers at levels above the discriminant level as an indication that there is suspicion of recurrence. It will be observed that the cut off values for Hp and Cp are considerably higher than the published 95th percentile in a "normal" population which are quoted as 2.2 and 0.45 g/l respectively [21]. On the other hand, AGP is at the 95th percentile of a normal population.

C-Reactive protein

After completion of the main results presented in this paper, a survey was made of the levels of C-Reactive protein. A level of >10 mg/l was used as a discriminant to identify patients outside the normal range (0–10 mg/l). The following results were obtained: primary tumours stages I–III 11/81 (13.5%): stage IV 11/26 (42%); overt metastases after mastectomy without treatment 7/24 (29%); follow-up mastectomy clinically tumour free 3/102 (2.9%); benign breast disease 0/55 (0%) and Controls 0/26 (0%). Eighteen out of the 32 positive results (56%) had evidence of at least one other acute phase reactant protein elevated above the discriminant level.

DISCUSSION

It is convenient to consider the tumour related antigens (CEA, βHCG and casein) separately from the acute phase reactant proteins and enzymes.

Tumour related antigens

There have been several studies of the levels of serum or plasma CEA encountered in breast cancer. Steward [2] found elevated CEA in 6/22 patients with primary breast cancer, without clinical evidence of distant metastases. The levels recorded were below 10 μg/l (Thomson [22] assay, cut off 2.5 μg/l/1) except in one instance of 15 μg/l associated with local spread. The incidence of positivity increased to 37/47 (78%) with metastatic disease, levels being scattered between 2.5 μg/l to >30 μg/l. Serial CEA measurements in metastatic breast cancer lead the authors to conclude that initial CEA levels before therapy had little bearing on the prognosis but that in general sustained normal CEA levels after therapy correlated with a good response.

Chu and Nemoto [1] using the Hansen assay [23] considered that CEA might not be an adequate monitor of chemotherapy because clinical inprovement in their series sometimes preceded fall in CEA levels. Borthwick *et al.* [3] detected elevated CEA levels in 55% of women with primary breast cancer; 38/74 fell in the range of 0.1–0.4 U/ml, but they also found that 27/200 randomly selected women had a detectable CEA level in this range; 3/74 of the primary breast cancer patients had circulating levels of >0.4 U/ml. In patients with advanced breast disease 40% had levels >0.4 U/ml whereas in benign disease 10% had detectable CEA values. When clinical response to antioestrogen therapy was assessed, 6/19 responders showed a distinct fall in CEA values while only 1/22 non-responders showed a fall over a 3 month period. Unfortunately 6/19 responders increased their CEA level which would have complicated the interpretation if encountered prospectively. Our results seem to support the general finding that a low level of incidence of apparent positivity on CEA assay is found in the sera from patients with primary breast cancer. The exact immunological relation between the material in the serum on breast-tumour derived extracts and colonic cancer-derived CEA awaits corroborative study.

The ectopic production of human chorionic gonadotrophin by non-trophoblastic tumours has been investigated by Braunstein *et al.* [4] and Gailani *et al.* [5] using radioimmunoassays specific for the β subunit of the glycoprotein. In breast cancer, elevated plasma levels in 4/33 and 6/20 patients were found by these workers respectively. In addition Braunstein *et al.* [4] established parallelism between the material in the plasma of a patient with adenocarcinoma of the breast and standard human chorionic gonadotrophin. The elution pattern on chromatography through Sephadex G-100 was also very

similar. Our assay for human chorionic gonadotrophin, specifically directed to the β subunit, has established transient slight elevations of human chorionic gonadotrophin in the serum of some patients with breast cancer but these are not sustained sufficiently to be of use in monitoring the disease.

The frequency of a raised K-casein in the serum of our series of patients is lower than that reported by Franchimont and his colleagues [6]. In particular there was a low frequency of elevation of this marker in the women presenting with stage IV tumour or at first discovery of metastatic cancer (4/34); this suggests it will not have wide application for the follow-up of the management of metastatic cancer. However, in a previous series from our laboratories we have shown that if the tumour is a K-casein producer its level in the blood can reflect responses to therapy [7].

Acute phase reactant proteins and enzymes

The non-specific response of APRPs in metastatic cancer is a well recognised phenomenon in tumours of a variety of different tissues of origin [15, 16]. Longitudinal studies have shown that the changes in the protein profile in an individual can provide useful information about the evolution of cancer, especially in those tumours where specific tumour markers are absent or occur in a limited portion of the disease bearing patients [15, 24]. However it is evident the variance of some of these proteins coupled with the phenotypic variation seen in Hp and AT may obscure their value as potential markers when studied in a vertical series as presented in this paper. Our data shows that the APRPs cannot help in the discrimination of benign breast disease from primary breast cancer (stages I–III). On the other hand patients presenting with stage IV disease frequently but not invariably have a disturbed plasma protein profile. The behaviour of alkaline phosphatase and gamma glutamyltranspeptidase indicates they are both helpful in monitoring metastatic cancer though inevitably their rise signals advanced metastases involving bone or liver or both sites. The normal distribution of the levels of these enzymes in women without evidence of metastatic cancer indicates they both have potential in the profile test for longitudinal studies as they appear to have fairly stable patterns in health. Such longitudinal studies made for several years after resection of bowel cancer indicate that the patient's own values of these enzymes tends to remain within a narrow range [25].

The battery of markers used in this study is clearly too insensitive to separate unequivocally non-malignant and malignant breast disease except in its advanced stages. Whatever marker or combination of markers are chosen the variance within the disease groups is such that from a single reading, with the exception of a proportion of women with metastatic cancer, little can be said about the clinical significance of the pattern. However, these studies do indicate there are significant trends in the proportion of the markers that become abnormal in the progression from localised to metastatic breast cancer.

In practical terms it is the long term post-operative surveillance of high risk groups, for example pre-menopausal women with stages II and III cancer, where monitoring may be used with the aim of identifying early recurrence or the assessment of the effect of adjuvant chemotherapy. If this is to be done, then the problem still lies in choosing the best combination of markers for the individual patient. This can be influenced partly by the spectrum of TRAs produced by the primary and aided by serial monitoring of APRPs especially using those without phenotypic variation such as AGP and Cp and preferably unaffected by oestrogens; antichymotrypsin has recently been shown to have these properties [26]. Clearly then it is the clinician who must make the decision as to whether he feels the lead time given by any monitoring system could seriously influence his choice of therapy. It still needs to be proven that monitoring could avoid subjecting women who are "cured" of breast cancer to aggressive adjuvant chemotherapy on the basis of statistical probability of recurrence. The tests, at 2–3 monthly intervals, would be a trivial burden to that of chemotherapy though to be of any real use they would have to provide a considerable lead time in the warning of recurrence compared to standard clinical investigations. The challenge to the scientists would then be to make sure the system is both effective and cost effective.

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Effects of Cytostatics on Proliferating and Stationary Cultures of Mammalian Cells

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Abstract—*The cytotoxic effects of dibromodulcitol (DBD), dianhydrodulcitol (DAD), vincristine (VCR), N-formyl-leurosine (F-LEU) and bleomycin (BLM) were studied on logarithmically growing and plateau-phase cultures of Chinese hamster cells. One hour treatment with DBD yielded sigmoid type survival curves. Synchronized cells were sensitive to the drug throughout the cell cycle, especially at late G_1. DBD killed rapidly growing cells at a higher rate than plateau-phase cells. DAD proved to be equally toxic to both types of cells. At DAD concentrations above* 15 μg/ml *plateau-phase cells were more sensitive than logarithmically growing cultures. The dose-dependent survival curves for DAD were of the same type as the DBD curves. VCR and F-LEU produced survival curves characteristic of phase-specific drugs, killing specifically S-phase cells in synchronous cultures. Plateau cells were more sensitive to VCR than log cells, while F-LEU especially killed log cells. BLM produced biphasic survival curves, damaging plateau-phase cells almost* 18 *times more effectively than log cells.*

INTRODUCTION

BETTER knowledge of the effects anticancer drugs have on cells should enhance the effective design of chemotherapeutic schedules. The tissue culture system is widely used for the experimental study of mechanisms of drug toxicity. While exponentially growing cell cultures provide information about the effects drugs have on proliferation kinetics, the action of drugs on non-cycling cells may best be studied on plateau phase cells.

Plateau cultures, in which a very slow cellular turnover occurs, may be better models for *in vivo* tumours [1, 2], hence the recent attention paid to this type of culture in evaluating anti-cancer drugs [3–7]. In this work we have studied the cytotoxic effects of antitumour drugs on logarithmically growing and synchronized cell populations and on plateau phase cultures of Chinese hamster cells.

MATERIALS AND METHODS

Cell culture

Clone 451 of a Chinese hamster cell line [8] has been carried in our Laboratory for over 10 yr in medium 199 (Flow Laboratories) supplemented with 10% fetal calf serum and incubated in a 5% CO_2 95% air atmosphere. The cells were subcultured three times a week to keep them continually in logarithmic growth. Under these conditions the cells had a cell cycle time of 16.5 hr, as determined by the labelled mitosis method [9]. The average length of the G_1 phase was 5.5 hr, S-phase 7.5 hr and the G_2 phase 3.5 hr. The population doubling time was 18 hr.

Cell synchrony

Synchronized cell populations were used to study the cell cycle phase specificity of the drugs. Synchrony was achieved by mitotic selection [10]. At least 93% of the selected cells were in the M-phase.

Induction of plateau cultures

Replicate Petri plates, 60 mm in diameter, were seeded with 2×10^6 cells each and incubated without changing the medium. The cell number, mitotic index and ^{3}H-TdR labelling index were determined daily in replicate cultures. The cultures were considered to have reached the plateau phase when the cell number became constant, the mitotic index was about 0.1% and between 1 and 4% of the nuclei were labelled. This was achieved by the second day of the culture.

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Table 1. *Cytotoxic agents studied*

Drugs	Source
Dibromodulcitol (DBD)*	Chinoin Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary
Dianhydrodulcitol (DAD)	Chinoin Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary
Vincristine (VCR)	Pharmaceutical Works of Gedeon Richter Ltd., Budapest, Hungary
N-formyl-leurosine (F-LEU)	Pharmaceutical Works of Gedeon Richter Ltd., Budapest, Hungary
Bleomycin (BLM)	Nippon Kayaku Co., Ltd., Tokyo, Japan.

*Abbreviations used throughout this paper.

Drugs

The agents studied are widely used in clinical practice, with the exception of F-LEU. This new Vinca alkaloid derivative is very promising in the treatment of malignant diseases of the hemopoietic organs, and unlike VCR has no neurotoxic side effects [11, 12]. The drugs and their manufacturers are listed in Table 1. Stock solutions were prepared in physiological saline, and diluted for use with physiological saline or with medium.

Dose–response experiments

Exponentially growing cells were treated with different concentrations of the drugs as follows: cultures were exposed for 1 hr to DBD, DAD and BLM, and for 3 hr to VCR and F-LEU. The drug was removed and the plates rinsed with Hanks' solution. The cells were then trypsinized and seeded into culture flasks at 500 cells per 25 cm^2 flask. The pH was adjusted with 5% CO_2 in air. After 8 days of incubation the medium was removed and the cells stained with a saturated solution of crystal violet 0.85% NaCl. Colonies containing more than 50 cells were counted under a dissecting microscope, and the surviving fraction (SF) was caluaculated as follows:

$$SF = \frac{\text{plating efficiency of treated culture}}{\text{plating efficiency of untreated culture.}}$$

The same procedure was applied to plateau cells. Late plateau cells on the fifth day after subcultivation were treated by adding the drugs to the exhausted medium. This was done to avoid growth stimulation by fresh medium.

The plating efficiency of untreated control cultures of logarithmically growing cells, late plateau phase cells and mitotically selected cells were 86, 76 and 66%, respectively.

RESULTS

Dose–response studies on plateau cultures and logarithmically growing cells

Survival curves of cells treated with DBD and DAD are shown in Figs, 1 and 2, respectively. Both drugs yielded sigmoid survival curves. As can be seen from Fig. 1, DBD was more toxic to logarithmically growing cells. DAD was equally toxic to plateau and proliferating cells at concentration up to 15 μg/ml. At higher drug concentrations, plateau cells were more sensitive (Fig. 2).

The survival curve of exponentially growing cells treated with VCR was biphasic. Survival dropped sharply at VCR concentrations up to 0.025 μg/ml. Plateau phase cells had a similar concentration-dependent survival curve, but showed a higher sensitivity to the drug than proliferating cells (Fig. 3). Figure 4 demonstrated the dose dependent sensitivity of asynchronous cells to the new Vinca alkaloid F-LEU. Survival decreased with increase in drug concentration, and reached a minimal constant level at F-LEU concentration of 150 μg/ml, thus indicating that the drug has specific toxicity to cells in a certain phase of the cell cycle. F-LEU was toxic to a lesser degree to plateau cultures.

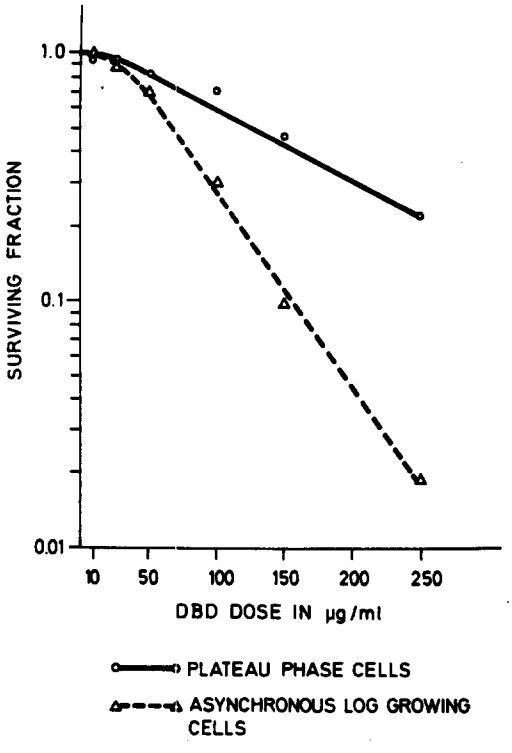

Fig. 1. Dose–response curve of Chinese hamster cells with DBD. Cells were exposed to different concentrations of the drug for 1 hr *after which the drug was removed and the cells washed with Hanks' solution. Cell survival was determined as described in "Materials and Methods."*

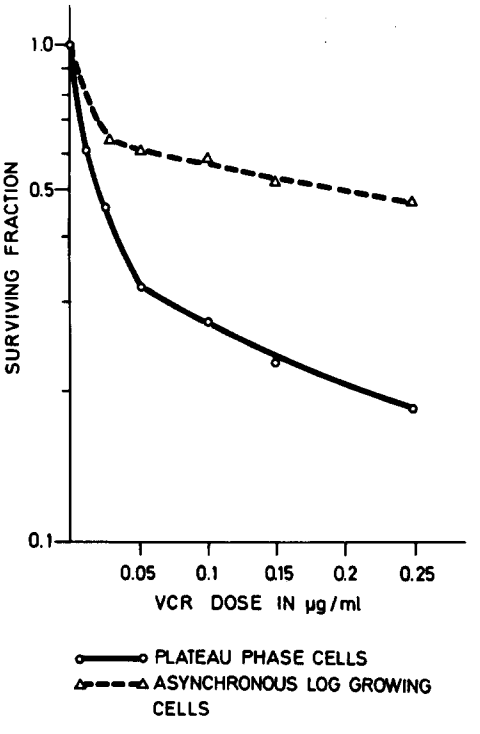

Fig. 3. Dose–response curve of Chinese hamster cells with VCR. Protocol was the same as in Fig. 1., except that the exposure to the drug was for 3 hr.

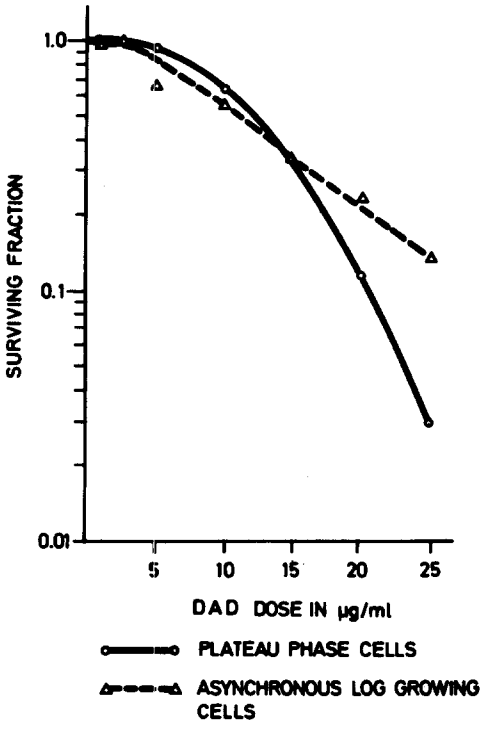

Fig. 2. Dose–response curve of Chinese hamster cells with DAD. Cells were exposed to different concentrations of drug for 1 hr. *Protocol was the same as in Fig. 1.*

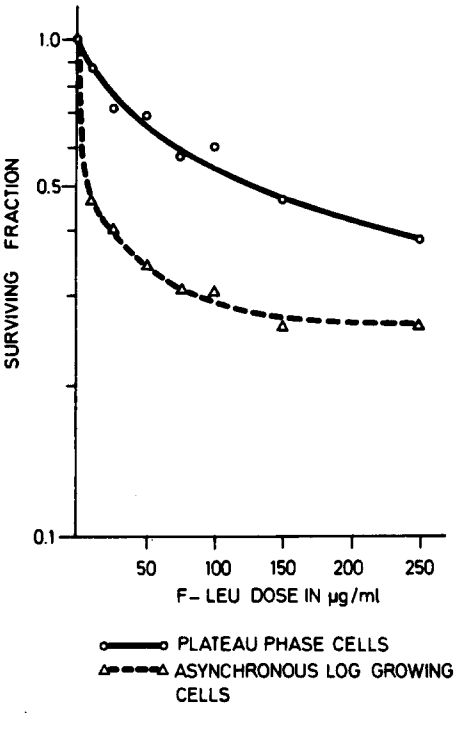

Fig. 4. Dose–response curve of Chinese hamster cells with F-LEU. Protocol was the same as in Fig. 3.

The sensitivity of plateau and logarithmically growing cells to BLM is shown in Fig. 5. Plateau phase cells were much more sensitive to the drug than proliferating cells. At a concentration of 100 μg/ml only 2% of the treated plateau cells survived compared to over 50% survival of the cycling cells.

Sensitivity of the synchronized cultures

The dose response of exponentially growing cells to F-LEU suggested that the drug has a phase specific toxicity as opposed to DBD, which judging by its survival curve should have no phase specific toxicity to cells.

Synchronized cultures were treated with DBD at 150 μg/ml for 1 hr. Similar treatment killed 90% of asynchronously growing cultures. As is shown in Fig. 6, DBD was toxic to cells throughout the cell cycle, but cells in late G_1 were more sensitive.

When synchronized cultures were treated for 30 min with F-LEU at 10 μg/ml a clear phase specific toxicity could be demonstrated. As is evident from Fig. 7, F-LEU was specifically toxic to cells in the S-phase, with little or no effect on cells in the other phases of the cell cycle.

DISCUSSION

We have tested the toxic effects of the alkylating agents DBD and DAD, the alkaloids VCR and F-LEU, and the antibiotic BLM on Chinese hamster cells. DBD showed a dose-dependent toxicity to both logarithmically growing and plateau phase cells, but proliferating cells were much more sensitive to the drug than cells in the plateau cultures. DAD affected cells from both types of cultures in essentially the same way, though at high drug concentrations plateau cells had lower survival. These results are in agreement with the experimental results of Lapis [13] who reported that *in vivo* low concentrations of both DBD and DAD were effective only on young NK ascites lymphomas. Only DAD at a high concentration inhibited old tumors.

When tested on synchronized cell populations, DBD exerted varied toxicity on cells throughout the cell cycle. Cells in the late G_1 phase were especially sensitive to the treatment, and showed a very low survival compared to cells in other phases or to logarithmically growing cells. These results confirm our preliminary experiments [14] where alterations in the nuclear DNA content and in the cellular ultrastructure were used as indicators of drug effect.

Our experiments with DBD clearly indicate that the drug is non-phase specific. The survival curve of cells treated with DAD

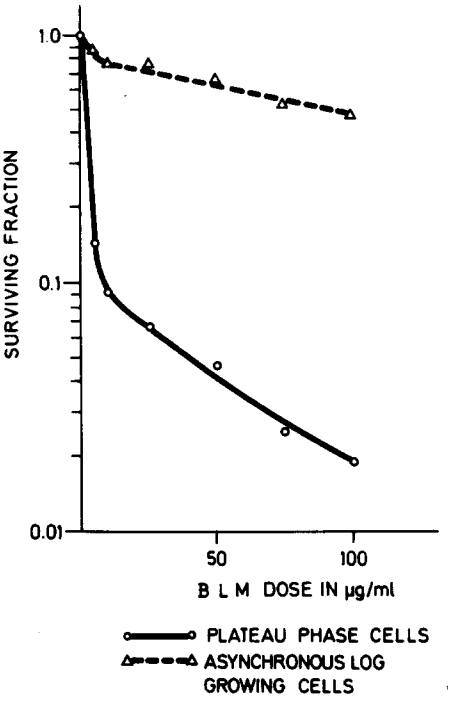

Fig. 5. Dose–response curve of Chinese hamster cells to BLM. Protocol was the same as in Fig. 1.

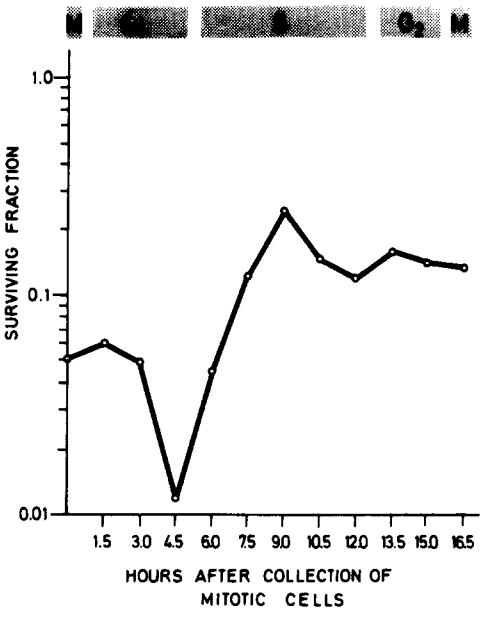

Fig. 6. Sensitivity of synchronous Chinese hamster cells to DBD (150 μg/ml). *Synchronized cells were exposed to the drug at* 1.5 hr *intervals after mitotic selection. The drug was removed after* 1 hr *and the cells washed with Hanks' solution. For each time point* 6 *replicate plates were used. Colonies were counted after* 8 days *of incubation. The variation in survival between the replicate plates was* 15%.

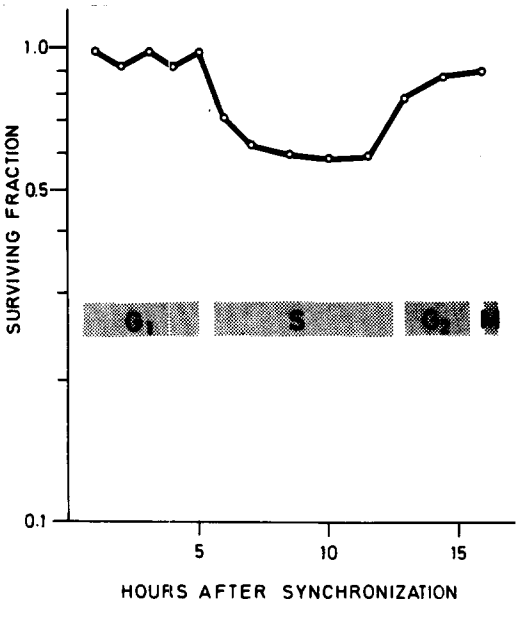

Fig. 7. Sensitivity of synchronous Chinese hamster cells to F-LEU. The cells were treated for 30 min *with* 10 μ/ml *and survival determined as described in Fig.* 6.

indicates that DAD too is non-phase specific. Palyi [15] and Barranco and Flournoy [16] arrived at the same conclusion when they reported that DAD was effective throughout the cell cycle with cells in S phase being the least sensitive. The different toxicity of DBD and DAD to logarithmically growing and to plateau phase cells cannot be attributed to altered binding to serum in fresh compared to "exhausted" medium, or to their stabilities for the duration of the treatment, but to the capacity of the cells to recover from the drug induced damage [16, 17]. Our results also strongly suggest that DBD and DAD differ in the mechanism of toxicity to cells, and that DAD is not the active metabolite of DBD [18].

Cells treated with the new Vinca alkaloid derivative, F-LEU, had a survival curve typical for phase specific drugs. When tested on synchronized cells F-LEU proved to be S-phase specific. Plateau cells were less sensitive to F-LEU. In contrast, non proliferating cells were much more sensitive to VCR, which is also an S-phase specific drug [19]. In all tested concentrations VCR had a much higher toxicity to plateau cells compared to proliferating cells. Rosner *et al.* [20] reported that, on long exposure to 20 μg/ml, VCR killed non-proliferating lymphoid cells at a higher rate than growing cells. In a previous report from this laboratory [21] we have found that clones of tumor cells of different biological characteristics exhibited different sensitivities to VCR and F-LEU.

The fact that these known mitotic spindle poisons are toxic also to non-proliferating cells may explain why metaphase-arresting ability is not necessarily associated with tumor inhibitory effect.

The antibiotic BLM was much more toxic to plateau cells than to proliferating cells. This difference in sensitivity cannot be attributed to binding of the drug in fresh medium [3, 5]. Barranco *et al.* [3] and Hahn *et al.* [6] have also reported that plateau phase cells are particularly sensitive to BLM. Twentyman and Bleehen [7] have treated fed plateau phase cells with BLM and found that the sensitivity of these sultures was largely dependent on the length of time the cells were maintained in the non-proliferating state. These experiments using fed plateau cells and our results from unfed plateau cells indicate that BLM is especially toxic to cells in the late plateau phase.

We observed great variability between the different drugs in regard to their toxicity to cells in proliferating and plateau cultures. Proper evaluation of cytotoxic effects of drugs thus requires testing on both exponential and plateau phase cell cultures.

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Prognostic Factors in Malignant Germ Cell Tumors of the Ovary in Children Excluding Pure Dysgerminoma

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Abstract—*Forty cases of malignant germ cell tumors of the ovary in children are presented, excluding pure dysgerminoma. They are classified according to the WHO classification.*

The histological typing and the stage have prognostic value, so they should be defined as accurately as possible.

The choice of therapeutic should take these two prognostic factors into account.

INTRODUCTION

AMONG the malignant germ cell tumors of the ovary in children, the pure dysgerminomas have a high rate of curability [1]. As this group represents another simpler problem in therapeutic management, they will not be included in this presentation. For the other malignant tumors of germ cell origin, the results of therapy remain poor [2–4]; the purpose of this study is to identify some prognostic factors in order to adapt the treatment.

MATERIALS AND METHODS

Population description

Forty girls with malignant germ cell tumors of the ovary, excluding pure dysgerminomas, have been evaluated in the pediatric department of our hospital between 1954 and 1976. There was no treatment administered, other than surgery, prior to the admission in our hospital. The mean interval between diagnosis and time of referral is 23 days. The median age at diagnosis is 11 yr.

The initial work up consisted of initial surgical procedure, chest radiography, and lymphangiography for the 26 most recent cases. Determination of alpha 1 feto-protein was done in the 22 most recent cases, prior to surgery or within the first post operative week, as the half life of alpha 1 feto-protein is approximately 6 days. Skeletal radiography, hormonal investigations, cytology of ascites fluid, liver scan, bone marrow examination were made when indicated. All patients were staged after the complete work up using a staging system inspired from Wollner [5].

Stage I included patients with disease limited to one ovary. Stage II included patients with disease limited to one ovary, and ipsilateral para-aortic nodes, and patients with bilateral primary tumor without pelvic extension, with or without para-aortic nodes involvement. Stage III included patients with disease spread to pelvis or abdominal wall, peritoneum, diaphragm, liver or other organs in the peritoneal cavity or patients with hemorragic ascitis or with clear ascitis with malignant cells. Stage IV included patients with distant metastases (lung, bones, brain, distant nodes).

The evaluation of intra abdominal extension is operatively and macroscopic. In 3 cases a second laparotomy was needed to assess the extent of abdominal tumor.

Histopathology

All slides were reviewed for this study; the number of blocks of tissue per case ranged from 6 to 30, the average being 13. The

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routine stain was Hematein-phloxin-Safran. In some cases, special stains (Gordon-Sweet PAS) were performed. The WHO histopathology classification was used [6], Figs. 1, 2 and 3.

Patients presenting immature teratoma associated with yolk sac tumor or with embryonal carcinoma were classified as yolk sac tumor or embryonal carcinoma.

Treatment

The treatment did not depend on pathology, but changed over time. Before 1967 the treatment of the primary tumor was surgery alone. From 1967 to 1970, a chemotherapy schedule similar to the Li protocol [7] has been systematically added to the surgery. It consisted of courses of MAC (methotrexate 7 mg/m^2/day × 5, actinomycin D 300γ/m^2/day × 5, cyclophosphamide 130 mg/m^2/day × 5) repeated every 3 months for 5 courses.

Since 1970 the protocol has been changed for patients with stage III and stage IV disease. For patients with stage III disease the protocol "OT 70" consists of one course of MAC followed by a radiotherapy: 4000 rad (Co 60) in five weeks four times a week on the whole abdomen without protecting the remaining ovary. The liver is blocked at 3000 rad and one or both kidneys at 1300 rad. With radiotherapy a specific diet is systematically administered [8]. After radiotherapy, courses of MAC are repeated every two months for four courses.

Patients with stage IV disease are treated with chemotherapy alone consisting of MAC with higher doses, bleomycin and adriamycin.

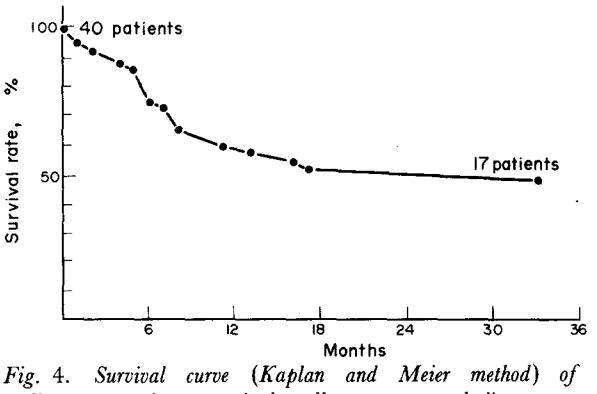

Fig. 4. Survival curve (Kaplan and Meier method) of malignant ovarian germinal cells tumors excluding pure dysgerminomas.

RESULTS

The origin of survival is the time of primary surgery. All the patients have at least a 24-month follow up and half of the patients have 7.5 yr follow up. Figure 4 shows the survival curve for the 40 patients.

The criteria chosen to test the prognostic value of stage and histology is the proportion of patients living with no evidence of disease two years after surgery, because the median of the time between diagnosis and local or distant relapse is 5 months, the latest relapse being observed 21 months after primary surgery. Table 1 gives the proportion of patients living with no evidence of disease for each stage and histology: it is different ($P<0.05$) between stage I and stage III and between yolk sac tumor, embryonal carcinoma and immature teratoma ($P<0.01$).

DISCUSSION

The survival of the population of children with malignant germ cell tumor of the ovary,

Table 1. Proportion of patients living with no evidence of disease after 24 months for each stage and histology (Living/Total)

	Stage (there was no stage II patients)			
Histology	I	III	Iv	Total
Yolk sac tumor*	1/2	1/11	0/2	2/15
Embryonal carcinoma†		5/10		5/10
Immature teratoma‡	7/8	3/4		10/12
Choriocarcinoma		0/3		0/3
Total	8/10	9/28	0/2	17/40

*1 patient with yolk sac tumor and immature teratoma. 1 patient with yolk sac tumor and dysgerminoma.
†2 patients with embryonal carcinoma and immature teratoma.
‡1 patient with immature teratoma and dysgerminoma.

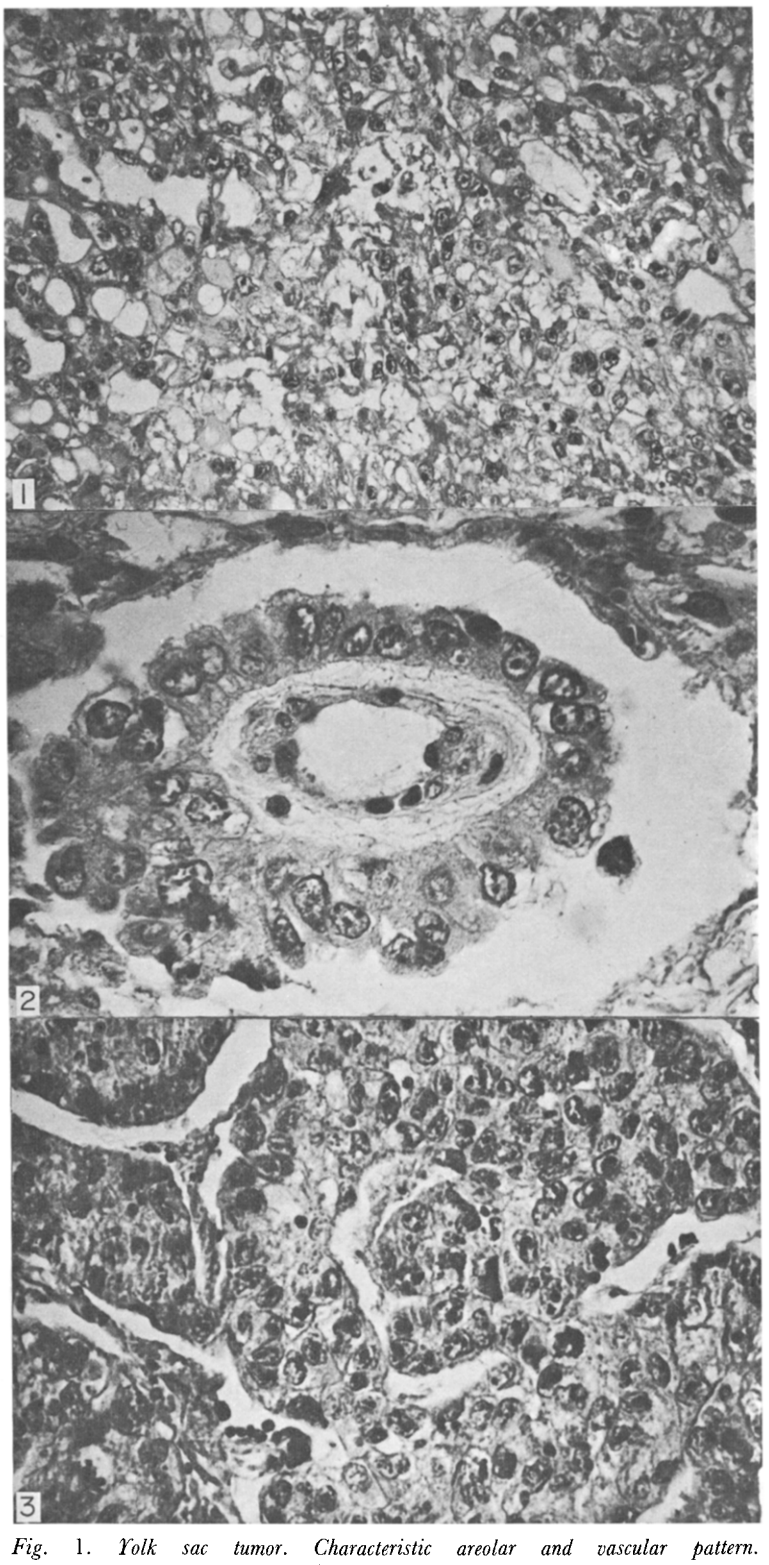

Fig. 1. *Yolk sac tumor. Characteristic areolar and vascular pattern.* (*H.P.S.* × 128).

Fig. 2. *Yolk sac tumor. Schiller Duval bodies. Note from the center to the outside: vessel stroma, epithelial structure with columnar cells, empty space and flattened cells.* (*H.P.S.* × 800).

Fig. 3. *Embryonal carcinoma: embryonal epithelial structures which are not considered as yolk sac tumor. Note the papillary and trabecular pattern; areolar and vascular structures are absent or difficult to find. There is no Schiller Duval bodies arrangements—the cytoplasm of the cells is dark or granular. In nuclei, nucleoli are prominent.* (*H.P.S.* × 128).

excluding pure dysgerminomas, studied here is similar to the survival reported by Wollner [5] on a comparable population. This study shows the prognostic value of stage and histology which should be defined as accurately as possible.

To determine the stage, the surgeon should examine the liver, peritoneal nodes, opposite ovary, uterus and remove all suspect elements for histologic examination. The child should be sent to a specialized service as soon as possible, this in order to avoid a second laparotomy.

In the determination of histology, immature teratoma and yolk sac tumor are well individualized entities but embryonal carcinoma is uneasy to define.

We classified immature teratoma according to the criteria defined by Thurlbeck and Scully [9]: there were 6 grades I, 4 grades II and 2 grades III tumors. We found a good prognosis of these tumors, as Norris [10], but their number is too small to assess the prognostic value of his grading.

Yolk sac tumor almost certainly originates from vitelline structures; however the term "endodermal sinus tumor" referring to a special vitelline structure of rodents [11, 12] is yet questionable; there are numerous intra and extra cellular hyalin globules containing alpha 1 feto-protein as demonstrated by immunofluorescence [13–15].

Embryonal carcinoma is a controversial entity; in the WHO classification there is no microphotograph and it is stated that it is an exceptional tumor in this organ; Kurman and Norris [16] have distinguished it from yolk sac tumor on histological and immunohistochemical characteristics and published 15 examples. Wollner *et al.* [5] defined embryonal carcinoma as "tumor growth showing densely packed undifferentiated neoplastic cells bearing amphophilic to basophilic cytoplasm". It is probably an heterogenic group, part of which could be undifferenciated yolk sac tumor, but further studies are needed.

Alpha 1 feto-protein dosage in blood by radio-immuno assay was done for the 22 most recent cases. For the 7 yolk sac tumors, alpha 1 feto-protein level was elevated. The other cases were negative, except one embryonal carcinoma and one immature teratoma; the patient with embryonal carcinoma had had chemotherapy prior to surgery and the tumor was necrotic at pathological examination. No yolk sac component was found on the slides available for the patient with immature teratoma. However, in our opinion, alpha 1 feto-protein secretion is a good indication of yolk sac tumor component.

For yolk sac tumor and embryonal carcinoma in adults and children, Kurman [17] observed different survival rates close to the rates reported here and noted the possible good prognosis of stage I disease.

The choice of therapeutic should take stage, pathology and alpha 1 feto-protein dosage into account. For immature teratoma and embryonal carcinoma a conservative surgery, that is to say unilateral oophorectomy, should be done on stage I patients, chemotherapy may even be unnecessary; for stage III patients radiotherapy associated to chemotherapy seems to have changed the prognosis: 6 patients out of 7 having received the OT 70 protocol are living with no evidence of disease two years at least after primary surgery.

For yolk sac tumor and choriocarcinoma, there are very few stage I patients, it is thus difficult to give a therapeutic indication; for stage III patients the disease progresses very quickly, short remissions were obtained with chemotherapy. Only more aggressive chemotherapy administered early might induce and prolong remission.

Acknowledgements—The authors thank Dr. Sarah Donaldson (Stanford University Medical Center, U.S.A.) for the translation of the manuscript into English. Dr. Buffe (CNRS, Villejuif, France) who performed the alphafetoprotein assays and the many surgeons who operated on the patients and referred them to us.

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Announcement

FIRST ANNOUNCEMENT: QUANTITATION OF TUMOUR RESPONSE: A CRITICAL APPRAISAL

The 9th L. H. Gray Conference will be held in Cambridge, England, during the week of 10–14 September, 1979. The topics of the meeting are intended to stimulate a critical appraisal of the techniques that are currently in use both experimentally and clinically for assessing tumour response after therapy. The topics will include:

In situ measurements of tumour response, including improved methods of tumour detection in clinical studies.
Factors of prognostic significance.
In vitro assays of tumours excised after treatment *in vivo*.
The choice of suitable experimental models in rodents.
The role of human tumour xenografts.
Statistical evaluation of tumour response data.
The limitations and artefacts of each assay technique.
The relationship between cell survival and the *in situ* response.

Further information from Dr. J. Denekamp, Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex, England.

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B. I. S. Srivastava, S. A. Khan, J. Minowada and A. Freeman: High terminal deoxynucleotidyl transferase activity in pediatric patients with acute lymphocytic and acute myelocytic leukemias.

N. Muñoz, R. J. L. Davidson, B. Witthoff, J. E. Ericsson and G. de-Thé: Infectious mononucleosis patients have an increased risk for Hodgkin's disease.

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R. mamoun, B. Guillemain, T. Astier, J. P. Portail, E. Legrand et J. F. Duplan: Production et analyse d'un complexe viral dèrivé d'un virus des radioleucoses de la souris C57Bl.

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Chemotherapy of Advanced Breast Cancer

Results of a Controlled Trial Comparing two Three-Drug Regimens

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Abstract—*Seventy patients with breast cancer in an advanced stage were treated, at random, according to one of the two regimens of cyclic administration, associating, on one hand, doxorubicin + vincristine + methotrexate and on the other hand, vincristine + cyclophosphamide + 5 fluorouracil. Only the immediate results are studied in this controlled trial in 61 evaluable patients. They are significantly better with the first association: 63% measurable objective responses vs 47.5% ($0.025 < P < 0.05$) and 30% of tumoral regressions superior to 50% vs 3.2% ($0.001 < P < 0.010$). The toxicity is hardly more marked (mainly cardiovascular and hematological) in the second regimen than in the first one, but remains altogether acceptable for an induction chemotherapy.*

INTRODUCTION

THE INTEREST of drug associations is generally recognized and has been particularly affirmed in the chemotherapy of breast carcinoma [1–3]. In the last few years a variety of therapeutic protocols of discontinuous application have been exposed and have been shown, regardless of the principle which guided their elaboration, very close in their effects as well as in their side effects.

Effectively, numerous publications give similar results, but they are difficult to compare and few randomized studies have been performed [4–8]. The announced percentages of objective global responses are usually superior to 50% with combinations of the drugs which are most often utilized: methotrexate, 5 fluorouracil, thiotepa, cyclophosphamide and vincristine.

The recent introduction of doxurubicin (adriamycin) in the therapeutic armamentarium along with the recognition of its efficacity in breast tumors [9, 10] has brought its insertion in new associations [11, 12]. This is what we have personally undertaken in a preliminary study which has rapidly given encouraging results. It thus becomes necessary to define the place of this combination chemotherapy by confronting its results with those obtained by the aid of the regimen that we have previously experienced (vincristine + cyclophosphamide + 5 fluorouracil) and which followed from the conclusions of comparative studies defining the most satisfying therapeutic index efficacy/tolerance [13].

The present controlled trial concerns only the determination, in a global manner, of the respective value of these two methods of treatment in order to assure further choice of a chemotherapy regimen to apply in priority for metastatic breast cancers. This analysis, limited in time, is available only for immediate effects and does not take into consideration the duration of the remissions or the influence on the survival of the patients

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(these long term effects should be ulteriorly studied). In any event, in the case of a favorable conclusion for the regimen including doxorubicin, a prolonged treatment is excluded because of the myocardic toxicity of the cumulative doses of this drug [14, 15] limiting it thus to an induction treatment.

This controlled trial was conducted from October 1st, 1975 to April 30th, 1976 in cooperation with 8 French Cancer Centers according to a commonly established study programme and identical means of data registration [16, 17].

MATERIALS AND METHODS

Treated patients

Seventy patients were included in this trial. The relative data are given in Table 1.

All included patients had either: a local relapse or an untreated breast cancer beyond surgery and radiotherapy; or a metastatic diffusion without any prior chemotherapy and without hormonotherapy of endocrine surgery since at least 3 months. All patients had at least one tumoral evaluable focus which allowed an objective evaluation of the efficacy. The precise histological diagnosis was established for all cases, with a predominance of differentiated adenocarcinomas (44 out of 70).

The main cause of exclusion (outside of the usual biological, psychological, familial or social conditions) was cardio-vascular history because of the known cardiotoxicity of doxorubicin. A balanced distribution was realized by randomization which lead to the

Table 1. *Characteristics of patient groups*

			Regimens	
		Total (70 cases)	VCF (35 cases)	DVM (35 cases)
Age	years: <40	5	3	2
	40–49	14	5	9
	50–59	22	12	10
	60–69	24	11	13
	≧70	5	4	1
	range	24–85	31–85	24–71
	median	60–62	58–62	60–64
Hormonal status:	premenopausal	9	4	5
	postmenopausal	61	31	30
Histology: adenocarcinomas:	differentiated	46	24	22
	indifferentiated	14	7	7
	anaplastic	3	1	2
	pleomorphical	1	1	0
	atypical	1	0	1
	unclassified	5	2	3
Interval between diagnosis and chemotherapy:	years: <1	19	7	12
	1–5	39	24	15
	>5	12	4	8
Prior treatments:	none	5	3	2
	surgery	52(3)*	30(2)	22(1)
	radiotherapy	61(11)	29(2)	32(9)
	hormonotherapy	21	9	12
Tumor sites:	breast	13	4	9
	regional lymph nodes	14	6	8
	metastatis sites	69	35	34

*Figures in brackets indicates isolated treatment.

Table 2. Treatment schedules

Days	CVF regimen	Days	DVM regimen
1, 2	vincristine: 0.6 mg/m²/day IV (without exceeding 1 mg by injection)	1	doxorubicin: 50 mg/m²/day IV
		2	vincristine: 1 mg/m²/day IV
3, 4, 5, 6	cyclophosphamide: 300 mg/m²/day IM + 5 fluorouracil: 500 mg/m²/day IV	3, 4, 5	methotrexate: 6 mg/m²/day (in 2 subcutaneous injections at 12 hr intervals)
Each cycle is discontinued for 25 days		Each cycle is discontinued for 15 days	

Table 3. Application modalities of chemotherapy

	Regimens		
	Total (70 cases)	VCF (35 cases)	DVM (35 cases)
Regimen applied: without modification	56	28	28
with modification	14	7	7
slight	8	3	5
important	6	4	2
Number of cycles: 1	9	4	5
2	20	12	8
3	17	11	6
4	8	4	4
5	5	2	3
6	7	0	7
7	4	2	2
Discontinuation of treatments: no	34	10	24
before 2 months	17	12	5
after 2 months	19	13	6
Causes: intolerance	6	2	4
(associated) failure	15	12	3
relapse	8	6	2
technical reasons	5	4	1
early death (before 2 months)	10	7	3

formation of 2 homogenous groups in function of the date given in Table 1.

Treatments

Each group of patients received a chemotherapy according to one of the regimens described in Table 2. The first one combines vincristine + cyclophosphamide + 5 fluorouracil (VCF), the second one doxorubicin + vincristine + methotrexate (DVM).

The follow up was identical for the two treatments and bore on the clinical state (especially neurologic and cardiovascular with an electrocardiogram before each cycle) and on the hematological state (the scheduled cycle was postponed for 8 days if the number of leukocytes was less than 3000 and/or the number of platelets less than 80,000).

The general modalities of application of each protocol are indicated in Table 3. The treatment was considered a failure after a delay of two months, unless there was an evident aggravation after one month. During this period, the CVF cycles were less numerous than DVM cycles, because the free intervals are larger in the first than in the second. The treatment was discontinued during the trial, especially for failure, 18 times with CVF regimen and 5 times with DVM regimen. Nearly half of the patients (34 out of

70) continued their chemotherapy until the date of analysis of the study.

Tolerance

The tolerance was studied for all patients of the two groups. The *clinical manifestations* of toxicity evaluated according to usual procedures [4, 7] were on the whole frequent; only 3 patients in each group did not present any side effects during the treatment. These manifestations were minor as indicated in Table 4: general reactions (asthenia), digestive (nausea and vomiting), cutaneomucous (alopenia, 21 times with CVF and 23 times with DVM; the latter in addition was responsible for 5 stomatitis). They generally responded correctly to symptomatic medications, but necessitated the discontinuation of the treatment in one patient submitted to CVF regimen and 3 patients submitted to DVM. Among these three last cases there was one cardiac ischemic attack (precordialgia and electrocardiographic signs); other cardiovascular disturbances particular to the DVM protocol because of doxorubicin were more mild: prolonged hypertension in one case; rhythmic disorder (tachycardia, extrasystoles) in two cases; electric abnormalities (repolarization disorders) in two cases. All in all the frequency and the intensity of the intolerance reactions were relatively more frequent with DVM than with CVF, but in any event, they were responsible for no death during chemotherapy.

The *hematological consequences* show little difference between the two regimens. The impairment was moderate, particularly for the erythrocytes, more important for the platelets and especially for the leucocytes. In one case a leukothrombopenia induced by the treatment (DVM) had been rapid and severe and caused infectious complications and lethal hemorrhages (the patient was in very poor condition when the treatment was started). No significant relationship was found between the toxicity and the number of cycles; with the prolongation of the treatment the tolerance was usually stationary and sometimes paradoxically better from the clinical as well as from the hematological point of view.

Table 4. Manifestations of intolerance and toxicity

	Regimens							
	VCF				DVM			
	IT1*	IT2	IT3	IT4	IT1	IT2	IT3	IT4
Global appreciation								
Number of cases	19	10	2	0	17	12	3	1
Manifestations: (associated)								
local	1	0	1	0	0	0	0	0
general	17	1	0	0	12	2	1	1
digestive	21	4	0	0	23	5	1	0
cardiovascular	0	0	0	0	3	2	1	0
epithelial	21	0	0	0	24	3	0	0
neurologic	1	0	0	0	1	2	0	0
hematologic:								
leukopenia	11	6	1	0	8	5	1	1
thrombocytopenia	4	1	0	0	3	4	1	1

*IT1: minor intolerance, without incidence on the treatment.
IT2: intolerance implying a modification of the treatment.
IT3: major side effects necessitating the discontinuation of the treatment.
IT4: toxicity leading to death.

RESULTS

The efficacy was judged subjectively and objectively.

1. *Subjective effects*

Forty-six evaluable cases of patients presenting at the beginning of the treatment either functional and/or subjective manifestations linked to tumoral evolution are divided into half for each group. The results are practically identical for the two types of treatment. The

numerical advantage is poor for DVM regimen (18 positive responses vs 15) but the antalgic effects of that association are particularly precocious, sometimes after the 1st cycle, bringing about a general subjective improvement which can compensate, in a very appreciable way, the manifestations of intolerance.

2. *Objective effects*

For 61 out of 70 patients of 2 groups having received a sufficient chemotherapy (minimum of 2 cycles) which permitted the analysis of the results, 46 or 75% (Table 5) manifested an objective response to the treatment evaluated by an estimation of the global tumoral mass (primitive tumor + lymph nodes + totality of the metastases) before and after treatment and codified by criteria already cited [4, 7]. The global difference observed between non responding and responding patients ($E \geqq 1$) is in favor of the DVM combination (response in 93% of the cases) and is statistically very significant ($P < 0.001$). A measurable regression of the lesions was registered in 29 cases (47.5%). It was obtained 10 times with CVF (32%) and 19 times with DVM (63%). The difference is again significant ($0.025 < P < 0.05$). Finally there is a distinctly higher proportion of regressions superior to 50% of the initial tumoral volume: 9 cases (30%) with DVM vs 1 (3.2%) with CVF ($0.0001 < P < 0.010$).

In global evaluation of responses, no complete regression was obtained, but the examination of the objective effects by tumoral sites (Table 6) shows 2 disparitions of pulmonary metastases, one in each group, and a total

Table 5. *Appreciation of therapeutic response*

Objective efficacity*	No. of the patients	Regimens VCF	DVM
E0	15	13	2
E1	17	8	9
E2	19	9	10
E3	10	1	9
E4	0	0	0
E9	9	4	5
Total	70	35	35

*E0: no response (progression).
E1: stabilisation or minimal regression $\leqq 20\%$.
E2: partial response $< 50\%$.
E3: partial response $\geqq 50\%$.
E4: complete regression.
E9: not evaluable for therapeutic response (early discontinuation or insufficient total dose).

Table 6. *Response of different tumoral sites*

Tumor sites	Regimens VCF (72 sites) E0	E1	E2	E3	E4	DVM (75 sites) E0	E1	E2	E3	E4
Breast	0	1	2	0	0	0	4	3	0	0
Regional lymph nodes	0	4	1	0	0	0	3	2	0	0
Metastases:										
lymph nodes	4	4	1	0	0	0	1	3	1	0
lung	4	6	1	0	1	1	3	3	1	1
liver	1	0	1	0	0	1	1	1	3	0
bone	5	5	2	0	0	1	9	1	0	0
skin	5	7	2	1	0	1	4	6	4	1
brain	0	1	0	0	0	0	0	0	0	0
pleura	3	4	1	0	0	1	5	1	1	0
opposite breast	2	1	2	0	0	1	2	5	0	0
Total	24	33	13	1	1	6	32	25	10	2
%	(33.7)	(45.7)	(18)	(1.3)	(1.3)	(8)	(42.7)	(33.3)	(13.3)	(2.7)

obliteration of cutaneous nodules in one case of DVM. Outside of the localizations generally responsive to chemotherapy (the same for primitive lesions and lymph nodes) there is an action particularly distinct in patients having hepatic metastases: 4 objective regressions with 3 of which superior to 50%, for 6 patients with an important hepatomegaly, along with concomitant retrocession of the jaundice which existed for 2 of them and the scintigraphic normalization in one case.

The first objective effects observed with DVM were frequently rapid (after the first and the second cycle) and were progressively reinforced to reach a maximum until the 5th or the 6th cycle.

The results in each group were significantly influenced neither by the age of the patient nor by the histologic type. The same was true for the hormonal status (genital activity or menopause). The delay of tumoral evolution (from the initial diagnosis to the beginning of the chemotherapy) does not substantially modify the quality of the responses obtained in the 2 series of patients.

DISCUSSION

The analysis of this controlled trial bearing on 2 groups of patients with advanced breast cancer, divided after randomization into similar groups and submitted to two different protocols of chemotherapy, showed a very significant advantage in favor of the association DVM: doxorubicin + vincristine + methotrexate, in discontinuous administration of 5 days cycles every 3 weeks. Even though the global immediate results are sensibly inferior to those of most other published studies (probably because of the differences in patient recruitment and the criteria adopted to evaluate the obtained effects), the homogenity of the conditons of the test allowed to bring about a useful comparison of the respective value of two applied therapies.

The side effects were discretely more frequent and more marked with the most efficacious association. They appeared nevertheless acceptable under reserve of indications correctly born and an attentive surveillance, especially cardiovascular and hematological. The total cumulative doses of doxorubicin should not however exceed 500mg/m^2, since beyond that limit the risk of myocardic toxicity augments substantially and does not authorize the continuation of the treatment. Consequently, the briefness of the cure should allow that chemotherapy only as a first sequence of treatment. This implies necessarily a continuation with one or several other protocols designed to maintain or to reinforce the obtained remissions.

It is a long term study of this type which has been now undertaken to determine the interest and the exact place of that association in more complex therapies, in order to improve the results of the palliative chemotherapy of advanced breast cancers.

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Results of a E.O.R.T.C. Randomized Trial of Cyclophosphamide and Radiotherapy in Inoperable Lung Cancer: Prognostic Factors and Treatment Results*†

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Abstract—*A randomized cooperative clinical trial has been conducted in inoperable carcinoma of the lung. One hundred and eighty-seven patients under age* 72 *of both sexes of all cell types were admitted provided they had had no previous surgery, chemotherapy or radiotherapy with the exception that patients having received less than* 2 g *of cyclophosphamide or less than* 2 weeks *of chemotherapy of any kind could be included. Treatment was assigned by randomization, stratified by institution and cell type, to one of the following treatments:* (1) *continuous radiotherapy* (6000 rad *at the rate of* 1000 rad *per week*), (2) *split-course radiotherapy* (3 *series of* 2000 rad *each administered in three* 2-week *periods separated by* 8-week *intervals*), (3) *continuous radiotherapy plus cyclophosphamide*, (4) *split-course radiotherapy plus cyclophosphamide. Cyclophosphamide was given at the dose of* 20 mg/kg *i.v. or i.m. every* 15 *days starting* 15 days *after the completion of continuous radiotherapy or* 15 days *after the first course of radiotherapy for those receiving the split-dose schedule.*

No statistically significant differences in survival were seen between treatment groups.

INTRODUCTION

THIS report describes the results of a randomized clinical trial conducted by the E.O.R.T.C. designed to answer two principal questions: (1) Does long-term chemotherapy improve survival rates for primary inoperable bronchogenic carcinoma treated by radiotherapy? (2) Is split-course radiotherapy better than continuous radiotherapy? At the time this trial was begun there was some evidence from radiobiologic experiments suggesting that split-course radiotherapy might produce longer remissions. Interest in this concept still exists [1–3], for although radiation has been used in the treatment of inoperable bronchogenic carcinoma for several decades, the optimal method of using this therapy has not been determined and reports in the literature are often difficult to evaluate because of very dissimilar prognostic categories and failure to make randomized comparisons. At the time this trial was designed the role of single agent chemotherapy in inoperable lung cancer was unclear. A previous controlled randomized trial of the E.O.R.T.C. Lung Cancer Cooperative Group [4] did not show any increase in survival in patients with limited inoperable disease treated with radiotherapy and concomitant short-term chemotherapy with cyclophosphamide compared to radiation therapy alone. The

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Group decided thereafter to implement another trial with long-term chemotherapy after radiotherapy. Although much more information is available on these subjects today, it is felt that all trials, whatever their results, should be published in order to provide a firm foundation for designing new trials. This is particularly true with respect to evaluating the role of prognostic factors and considerable attention will be devoted to that subject in this report.

MATERIALS AND METHODS

Between November 1968 and August 1971 patients from 12 hospitals in Holland, Belgium, France and Italy were admitted to this study.

In order to be eligible for the trial patients had to have primary bronchogenic carcinoma either histologically confirmed or with sufficient presumptive evidence (cytology and clinical presentation) that the patient was treated as having that condition.

Patients under age 72 of both sexes and of all cell types were admitted provided they had had no previous surgery, chemotherapy or radiotherapy with the exception that patients having received less than 2 g of cyclophosphamide or less than 2 weeks of chemotherapy of any kind could be included. Patients having received chlormethine (nitrogen mustard) were excluded no matter what the dose had been. In addition, the clinical stage was required to be such that radiotherapy was indicated, that is, limited inoperable lung cancer. Other criteria for exclusion included cavitary forms, massive tumors which could not be contained in the field of irradiation, that is, exceeding 225 cm^2 (15 × 15), those cases with significant pleural effusion which was cytologically positive, patients with metastases with the exception of mediastinal metastases which could be included in the field of irradiation and patients having other primary cancers with the exception of cutaneous epitheliomas previously cured or curable. All histological forms of lung cancer were considered eligible for the trial. Patients were required to have clinical and hematological status which would permit aggressive treatment with radiotherapy and chemotherapy. Finally, the patients' sociopsychological condition was required to be such that the physician treating them could expect that the treatment would be followed regularly and completely.

Treatment was assigned by randomization stratified by hospital and cell type (epidermoid carcinoma, anaplastic carcinomas—including small cell and undifferentiated carcinomas—and other) to 1 of 4 groups: a single series of continuous radiotherapy (R10), continuous radiotherapy plus cyclophosphamide (R1C), split-course radiotherapy in 3 series (R30), or split-course radiotherapy plus cyclophosphamide (R3C). The schema is given in Fig. 1. Continuous radiotherapy consisted of 6000 rad tumor dose given at a rate

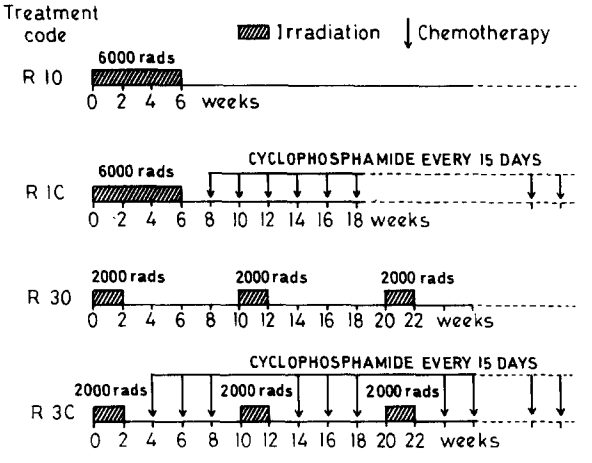

Fig. 1. Schema of the study.

of 800–1000 rad per week for a total duration of about 6 weeks. The irradiated area was not allowed to surpass 225 cm^2 (15 × 15). The radiotherapy was given by a high energy source, either cobalt or betatron. The split-course radiotherapy was given in 3 series of 2000 rad each administered over 3 two-week periods separated by 8-week intervals giving a total treatment duration of about 22 weeks. Remarks concerning the area of irradiation and the type of radiotherapy are the same as for the continuous radiotherapy.

Chemotherapy consisted of cyclophosphamide administered in a dose of 20 mg/kg or 700 mg/m^2 of body surface area not to exceed 1200 mg given i.v., or intramuscularly if intravenous injection was not possible. This therapy was repeated as a single injection every 15 days. Treatment began 15 days after the completion of radiotherapy for the group receiving continuous radiotherapy and 15 days after the completion of the first course for those receiving split-course radiotherapy. As shown in Fig. 1, the patients receiving split dose radiotherapy received 3 injections of cyclophosphamide between courses, but did not receive chemotherapy during courses of radiotherapy. If the white cell count was less than 4000 or the platelet count less than

100,000 the interval between doses was increased by 1 week without diminishing the dose of cyclophosphamide. If the leukocyte count and platelet count did not exceed these values at the end of one week, further 1 week waiting periods were added as necessary without diminishing the doses of cyclophosphamide. If the white cell count fell below 2000, radiotherapy was interrupted for a period not to exceed 2 weeks. If this period was insufficient for recovery of the white cell count to a value of greater than 2000 the treatment was terminated. If the tumor became worse in the group receiving split-course radiotherapy without chemotherapy in the interval between 2 series, radiotherapy was reinitiated or other therapy could be given provided the reasons were stated. Other treatments before radiotherapy were not permitted with the exception of atropine and antibiotics. Corticoids were specifically prohibited except in cases of absolute necessity.

Patients receiving continuous radiotherapy were examined every week for the first 6 weeks. For those receiving split-course radiotherapy, the examinations at the third and fifth week could be omitted. Between 6 and 12 weeks patients were examined every 2 weeks and from 12 to 20 weeks every 4 weeks. After 20 weeks patients were examined every 6 weeks for the rest of the study. The principal criteria of evaluation were time until recurrence or metastasis and survival. Performance was evaluated according to the criteria of Karnofsky [5]. Additional treatment could be introduced as indicated at the time of recurrence or metastasis.

The analysis presented here describes the results for 187 patients for whom initial data, details of treatment and adequate follow-up were available. Before analyzing the data detailed computer editing was performed to detect coding errors and to check the consistency of the data. Errors were resolved by checking the original data forms. Survival curves were constructed using the actuarial method [6] and tested for significance using the Mantel–Haenszel technique [7]. The effect of prognostic variables on response rates were examined using logistic regression techniques (see Appendix) and the effect of prognostic factors on survival were examined using a mathematical model incorporating covariate information based on the Weibull distribution (see Appendix).

RESULTS

Treatment received

Patients assigned to continuous radiotherapy were more likely to receive a full dose of radiotherapy compared to those assigned to split-course therapy (Table 1). For the 2 groups receiving continuous radiotherapy combined, 84% received between 5500 and 6500 rad compared to 55% for the 2 groups on split-course radiotherapy combined. Patients assigned to split-course radiotherapy were, of course, at greater risk of recurrence, metastasis, or death before completing their course of radiotherapy and, in some instances, they may have failed to return to the clinic to complete their therapy. Despite the striking difference between the number of patients receiving a full course of radiotherapy, all survival curves are based on the initial assignment to treatment groups. It would be incorrect to delete the patients in the split-course groups who did not receive the full course of therapy since this would bias the comparisons in favor of the split-course radiotherapy by eliminating the patients who experienced early recurrence, metastasis or death.

Table 1. *Dosage of irradiation received by treatment group*

	Treatment				
Dosage (rad)	R10	R1C	R30	R3C	All Treatments
≧6500	0	0	0	0	0
5500–6499	45	42	20	26	133
4500–5499	5	4	1	1	11
3500–4499	2	2	8	6	18
2500–3499	2	0	0	0	2
1500–2499	0	0	9	11	20
<1500	1	0	0	0	1
Unknown	1	0	0	1	2
Total patients	56	48	38	45	187

The 2 groups of patients receiving chemotherapy were reasonably comparable with respect to the average number of injections received, the average total dose, the minimum values of platelets observed during treatment and the minimum white blood cell counts (Table 2). Other than the effects on the hematological system, the chemotherapy produced no unusual toxicity and weakness, nausea and diarrhea were only rarely reported.

Prognostic factors

A large number of variables concerning the patient's status at the time of entry to the trial were recorded. These included previous therapy, various symptoms, performance status (Karnofsky score), cell type, size of the tumor and other variables related to the radiological appearance of the tumor (such as the presence or absence of atelectasis, the location of the tumor and the nature of the tumor image). In order to determine which of these variables most affected survival, death rates (defined as the number of deaths per 100 patient months of follow-up) were computed for each variable at its different levels. Those variables showing definite prognostic significance are shown in Table 3. Since these variables are likely to be interrelated, they will not all be needed to assess prognostic significance, and in a later section a summary analysis will be presented. However, the treatment groups may be compared with respect to these variables known to influence prognosis. Such an analysis is presented in Table 4 along with χ^2 statistics testing whether the distributions of these variables differ according to treatment group. In all cases the test statistic is non-significant indicating that the randomization procedure was effective in producing comparable treatment groups.

In addition to the variables listed in Table 3 other variables were recorded which concerned the radiological appearance of the tumor. These included the presence or absence of atelectasis, a characterization of the tumor image as complex or non-complex, information on the portion of the total pulmonary field occupied by the tumor and the location of the tumor. Some of these variables showed a definite relationship to prognosis. We mention this fact to encourage investigators to record information on atelectasis, size and location of tumors in future studies.

Age was not a significant prognostic factor in this study. The age range was 39 to 77 (2 patients over age 72 were admitted to the trial, 1 aged 73 and 1 aged 77). The median age was 61 and the age distribution was somewhat skewed toward the younger age group with mean 60.1 and standard deviation 7.3. There were 180 male patients and 7 female patients, so the data were inadequate to assess sex as a prognostic factor.

We were particularly interested in the results of the tuberculin test as a prognostic factor since in previous reports [8] it had been suggested that a negative tuberculin test was associated with diminished survival and that a negative reaction may become positive after the removal of the primary tumor. Patients in which this has happened have experienced particularly long survival. In our study tuberculin tests were performed in 62 cases of which 16 were negative and 46 were positive.

Table 2. Comparison of the two groups receiving chemotherapy

	Treatment	
	R1C	R3C
Number of patients	48	45
Average number of injections ±S.E.	7.81±0.93	6.73±0.66
Average total dose in mg ±S.E.	7793±1002	7429±749
Minimum white blood cell count		
>6000	6	8
5000–5999	8	4
4000–4999	10	13
3000–3999	9	13
2000–2999	4	1
<2000	0	2
Unknown	11	4
Minimum platelet count		
>200,000	9	15
150,000–199,000	10	13
100,000–149,999	13	7
<100,000	4	4
Unknown	12	6

Table 3. Variables showing prognostic significance

Variable		Number of patients	Number of deaths	Total months follow-up	Death rate per 100 patient-months
Previous antibiotic therapy	No, Unknown	117	108	1365	7.91
	Yes	70	63	504	12.50
Weight loss	No, Unknown	74	67	881	7.60
	Yes	113	104	988	10.53
Anorexia	No, Unknown	130	119	1509	7.89
	Yes	57	52	360	14.44
Weakness	No	94	89	1131	7.87
	Yes	93	82	738	11.11
Dyspnea	No	80	71	907	7.83
	Yes	107	100	962	10.40
Dysphonia	No, Unknown	154	138	1679	8.22
	Yes	33	33	190	17.37
Performance status	90–100, Unknown	90	80	1113	7.19
	70–80	75	70	619	11.31
	20–60	22	21	137	15.33
Cell type	Epid., Other	144	129	1565	8.24
	Anaplastic	43	42	304	13.82
Size of tumor	≦5 cm, Unknown	115	104	1280	8.13
	>5 cm	72	67	589	11.38
All patients		187	171	1869	9.15

Table 4. Distribution of patients for important prognostic variables by treatment group

Variable		Treatment group R10	R1C	R30	R3C	Chi-square (degrees of freedom) *P*-value	
Previous antibiotic therapy	No	30	23	20	25		
	Yes	20	19	15	16	1.012	0.985
	Unknown	6	6	3	4	(6)	
Weight loss	No	24	17	12	15		
	Yes	29	30	24	30	5.016	0.542
	Unknown	3	1	2	0	(6)	
Anorexia	No	36	31	27	34		
	Yes	19	17	10	11	4.103	0.663
	Unknown	1	0	1	0	(6)	
Weakness	No	28	23	21	22	0.521	0.914
	Yes	28	25	17	23	(3)	
Dyspnea	No	26	16	19	19	2.869	0.412
	Yes	30	32	19	26	(3)	
Dysphonia	No	42	42	28	40		
	Yes	13	6	10	4	8.116	0.230
	Unknown	1	0	0	1	(6)	
Performance status	90–100	22	21	12	18		
	70–80	24	17	18	16	4.007	0.911
	20–60	6	7	4	5	(9)	
	Unknown	4	3	4	6		
Cell type	Epidermoid	25	23	15	27		
	Anaplastic	14	10	12	7	4.989	0.545
	Other, Unknown	17	15	11	11	(6)	
Size of tumor	≦5 cm	24	21	21	16		
	>5 cm	23	21	12	16	7.317	0.293
	Unknown	9	6	5	13	(6)	
Total patients		56	48	38	45		

The death rate among the negative cases was 14.61 per hundred patient months vs 8.89 for the positive cases. An F-test comparing these two death rates gave a *P*-value of 0.079. Although this result is not convincingly significant, possibly because of the small number of cases, it is in the direction reported in other studies suggesting a more favorable response for patients who had positive tuberculin tests. However, we have examined the relationship of the tuberculin test result to three other prognostic variables, the presence of dyspnea, the presence of an anaplastic tumor, and the presence of weakness. Patients with a negative tuberculin test were 2.75 times more likely to have dyspnea, 2.74 times more likely to have an anaplastic tumor and 7 times more likely to have weakness. We are therefore hesitant to draw conclusions about the meaning of tuberculin test results. It may be that the prognosis is worse simply because as the tumor becomes more advanced the test becomes negative. The alternative explanation would be that the tumor becomes more advanced because the tuberculin test is negative, demonstrating that the patient has diminished immunologic capacity. Further studies will be required to resolve this issue.

Using covariates to predict survival

In order to study the relationship between the various prognostic factors shown in Table 3 a parametric survival model incorporating covariate information was used (see Appendix). The model chosen is based on a Weibull distribution in which the death rate is a function of the initial characteristics of the patients; this model permits the death rate to increase or decrease as a function of time. If the death rate is constant over time, then survival is governed by an exponential distribution which is a special case of the Weibull distribution. Details of the fitting procedure are given in the Appendix. In this study all of the variables listed in Table 3 were entered into the model. All variables were coded 0, 1 (except Karnofsky's score which was coded 0, 1, 2) in order of death rates and then systematically removed until the fit of the model

Table 5. Characteristic of the risk groups and coefficients for Weibull model

Group	Karnofsky	Anorexia	Dysphonia	Number of patients	λ_i*	Predicted survival at 1 yr		Observed survival at 1 yr
I	0	0	0	62	2.134	0.542		0.581
II	1	0	0	43	3.723	0.343		0.334
III	0	1	0	18	4.360	0.286		
	2	0	0	7	5.312	0.218	0.226†	0.189
	0	0	1	6	6.096	0.174		
	1	1	0	16	5.949	0.181		
IV	1	0	1	8	7.685	0.110		
	2	1	0	8	7.538	0.115		
	0	1	1	4	8.322	0.092		
	2	0	1	4	9.274	0.070	0.086†	0.114
	1	1	1	8	9.911	0.058		
	2	1	1	3	11.500	0.037		
All patients				187				

Regression coefficients for Weibull model

		β‡	S.E.‡	β/S.E.
Underlying force of mortality	1 (All patients)	2.134	0.572	3.371
Karnofsky rating	0 90–100, Unknown			
	1 70–80	1.589	0.559	2.843
	2 <70			
Anorexia	0 Absent, Unknown	2.226	0.980	2.271
	1 Present			
Dysphonia	0 Absent, Unknown	3.962	1.552	2.553
	1 Present			

*Death per patient-month × 100.
†Weighted average.
‡Times 100.

to the observed data was significantly affected. This procedure allows us to study the interrelationships of the prognostic variables since each can be examined separately while adjusting for the effects of the other variables. We found that survival could be adequately predicted using only three of these variables, the Karnofsky score, the presence or absence of anorexia and the presence or absence of dysphonia. The Karnofsky score may be thought of as an overall measure of patient's status while anorexia is probably most closely correlated with cachexia from progressive disease and dysphonia probably indicates patients in whom the tumor has spread to the mediastinum. Using these three variables we defined four risk groups based on increasing values of the predicted death rate for individual patients. The characteristics of these risk groups are shown in Table 5. The patients in the first risk group had normal levels for all three variables. Those in risk group 2 were characterized by Karnofsky scores of 70–80 in the absence of unfavorable values for the other two variables. Patients in risk group 3 tended to have abnormal values for anorexia or dysphonia or a Karnofsky score less than 70. Patients in risk group 4 always had abnormal values for at least two of the three variables. Also shown in Table 5 are observed and predicted survival figures at 1 yr for the four risk groups and the values of regression coefficients for the model. There appears to be reasonable agreement between the model and observed data. The plot of observed and predicted survival for the four risk groups is shown as Fig. 2. This plot is on the logarithmic scale. The fact that all the lines bend downward indicates that the death rate is increasing with time and justifies fitting the Weibull model. The value of the shape parameter was 1.351. If the death rate had been constant in time the value of the shape parameter would have been near 1.00.

Tumor response

Although survival is of paramount interest in a study of advanced lung cancer, the response of the tumor may give clues that might lead to improved therapy. Variables which are important in predicting survival may differ from those useful in predicting response to treatment and must be examined separately. In this study tumor response was designated as slight, moderate or marked. For this analysis we have grouped all responses together. The first question of interest was to examine response by cell type. Results of this analysis are given in Table 6. Despite some variability in the table, there are no significant differences between the response rate for the four therapies within any given cell type. The overall response rate was about 66% and patients who responded to therapy survived significantly longer than those who did not ($P < 0.02$). In order to predict response to therapy we examined many possible prognostic

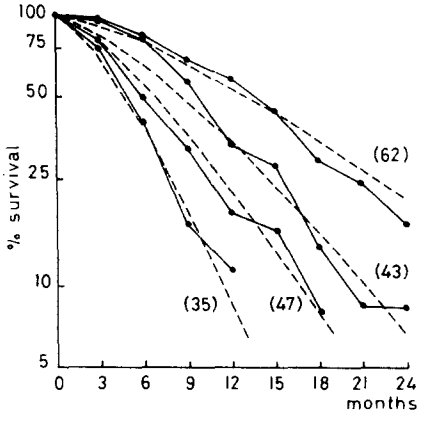

Fig. 2. Observed (——) and predicted (- - -) survival for the 4 risk groups. ( ) Number of patients.

Table 6. Response rates by treatment and cell type

Treatment	Cell type			
	Epidermoid	Anaplastic	Other and unknown	All cell types
R10	0.560 (14/25)	0.500 (7/14)	0.706 (12/17)	0.589 (33/56)
R1C	0.652 (15/23)	0.700 (7/10)	1.000 (15/15)	0.771 (37/48)
R30	0.600 (9/15)	0.583 (7/12)	0.727 (8/11)	0.632 (24/38)
R3C	0.667 (18/27)	0.571 (4/7)	0.636 (7/11)	0.644 (29/45)
All treatments	0.622 (56/90)	0.581 (25/43)	0.778 (42/54)	0.658 (123/187)

*There were 9 patients with unknown cell type, 2, 1, 3 and 3 in treatment groups R10, R1C, R30, R3C respectively.

Table 7. Predicted and observed response rates based on logistic regression using three prognostic variables

Involvement of recurrent laryngeal nerve	Tumor size >5 cm or unknown	Poorly differentiated epidermoid lesion	Number of patients	% response Obs.	% response Pred.*	No. responding† Obs.	No. responding† Pred.
−	−	−	64	82.8	82.1	53	53
−	−	+	9	55.6	58.1	5	5
−	+	−	73	67.1	66.2	49	48
−	+	+	16	31.3	37.1	5	5
+	−	−	8	62.5	61.4	5	5
+	−	+	1	0.0	32.5	0	0
+	+	−	13	30.8	40.4	4	5
+	+	+	3	66.7	17.0	2	1
All patients			187	65.8		123	122

*The fitted prediction equation was $ln(p/(1-p)) = 1.523 - 1.059\ \chi_1 - 0.852\ \chi_2 - 1.197\ \chi_3$ where χ_j, $j = 1, 2, 3$ takes the value 1 if the unfavorable factor is present and 0 otherwise. χ_1 = involvement of recurrent laryngeal nerve, χ_2 = tumor size >5 cm or unknown and χ_3 = poorly differentiated epidermoid lesion. The T-values (regression coefficients divided by their standard errors) for the three variables were −2.32, −2.51 and −2.78 respectively.
†Rounded to nearest whole number.

factors. After eliminating variables not needed, we found that response to therapy could be adequately predicted with three variables: involvement of the recurrent laryngeal nerve, tumor size greater than 5 cm or tumor size non measurable and poorly differentiated epidermoid lesions. These variables were used in a logistic regression equation to predict survival (see Appendix). The results of our predictions compared to the observed responses (Table 7) indicate that the model fits the observed data quite well. However, it should be noted that we have examined a large number of variables in obtaining these three and thus we are not able to assess the true significance level for these variables since we are fitting the model to the same data used in estimating the coefficients. However, it is reasonable to identify these variables as being importantly related to response to treatment. Response to treatment was less likely if there was involvement of recurrent laryngeal nerve, if the tumor was greater than 5 cm or was not measurable, or if the lesion was of the poorly differentiated epidermoid type on histological examination. It is probable that the tumor was not measurable because of its infiltrative character or the presence of atelectasis which makes it impossible to measure the tumor accurately. For this reason tumors of unknown size behave like large tumors.

Treatment comparisons

Survival curves for the four treatment groups are shown in Fig. 3(a) and the median survival times are given in Table 8. It is apparent from examining this Figure that there were no significant differences in survival between the four treatment groups and this impression is confirmed by formal significance tests. In Fig. 3(b) survival of patients receiving continuous radiotherapy is compared with the group receiving split-course radiotherapy. Again the difference is not significant. In Fig. 3(c) patients receiving chemotherapy are compared with those who did not receive chemotherapy. Again there are no significant differences. In Fig. 3(d) survival curves are shown according to cell type. As has been reported in many other studies, patients with epidermoid or other kinds of bronchial carcinoma survive longer than patients with anaplastic tumors. There was no significant difference between epidermoid carcinomas and other histologic types with the exception of anaplastic tumors. In these treatment comparisons all patients have been included whether or not they received a complete course of their assigned therapy. This policy is necessary in order to avoid introducing serious biases in analyzing the data. Nevertheless parallel analyses were done using only patients who received a full course of radiotherapy or who received 80% or more of their intended dose of chemotherapy. These analyses did not reveal any significant differences between the four treatments.

Although data were often incomplete on Karnofsky scores as treatment progressed, the available data were studied to see if the quality of life might be better for some treatments compared to others. However, no significant differences were noted. In addition, the data were examined to see if there were differences in survival by treatment group

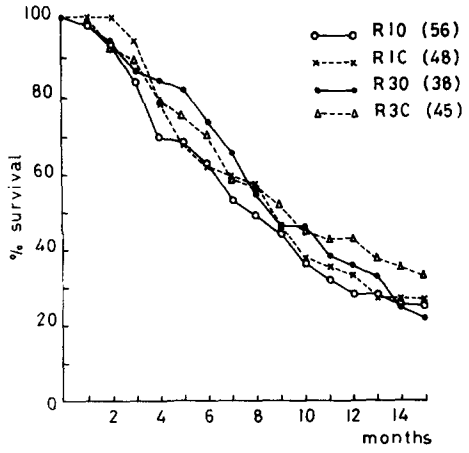

Fig. 3(a). Actuarial survival curves by treatment.

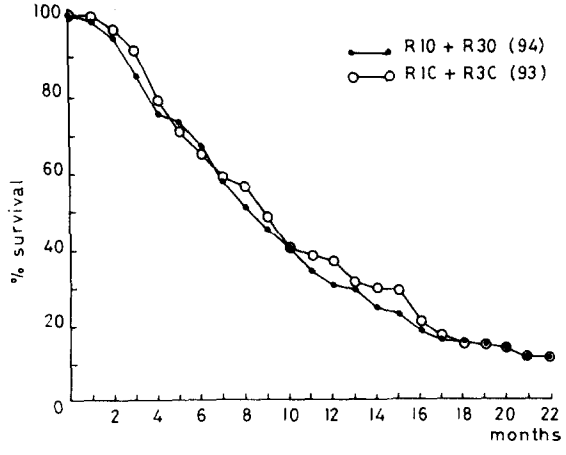

Fig. 3(c). Actuarial survival curves comparing chemotherapy with no chemotherapy.

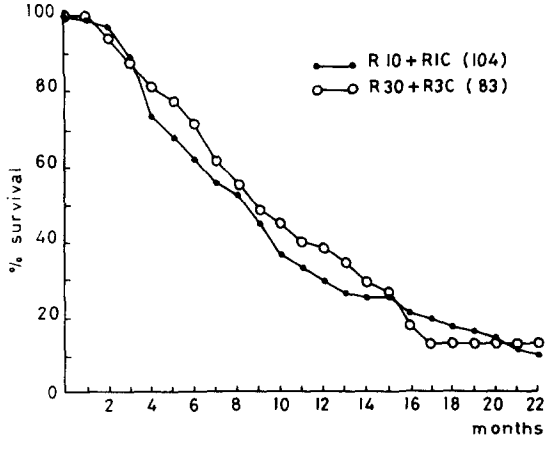

Fig. 3(b). Actuarial survival curves by continuous with split course radiotherapy.

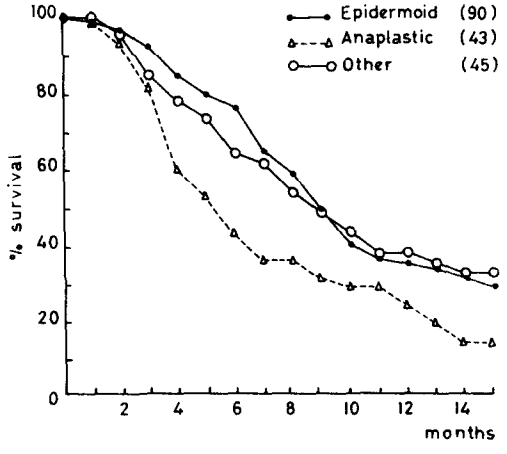

Fig. 3(d). Actuarial survival curves by cell type.

within the three major cell types. The numbers available for these analyses were small and no significant differences were detected.

DISCUSSION

In this analysis we have demonstrated the importance of several prognostic factors, both for response to treatment and for survival, in patients with advanced bronchogenic carcinoma. The fact that these variables have such an important effect on either response rates or survival rates indicates the desirability of conducting randomized clinical trials if meaningful treatment comparisons are to be made in patients with advanced lung cancer.

The multivariate methods used in this study both for prediction of survival (the Weibull model) and for the prediction of response to therapy (the logistic regression model) will be useful in analyzing other studies characterized by marked heterogeneity of patients. These techniques allow adjusted treatment comparisons in non-randomized studies and may be employed to refine treatment comparisons in randomized ones. In addition they may teach us valuable lessons about the natural history of the disease.

No treatment comparisons in this study were significant. The median survival time (9 months) for patients with epidermoid carcinoma are close to the ones observed in other studies utilizing radiotherapy with or without chemotherapy [1, 2, 9]. The absence of improvement with cyclophosphamide confirms the results of several other randomized trials [10–12] including the previous trial of our own group with short-term chemotherapy [4]. Only one randomized trial has shown an increase in survival over radiotherapy alone in patients treated with cyclophosphamide and radiation therapy [13]. In this latter study chemotherapy was also given intermittently but at higher doses. Bleomycin plus radiotherapy was found superior to radiotherapy alone, in terms of survival, in a controlled

Table 8. Median survival time

Treatment	Number of patients	Median survival* Months	Weeks
R10	56	7.743	(30.97)
R1C	48	8.653	(34.61)
R30	38	8.534	(34.14)
R3C	45	9.215	(36.86)
All treatments	187	8.565	(34.26)
Cell type	Number of patients	Median survival Months	Weeks
Epidermoid	90	8.933	(35.73)
Anaplastic	43	5.306	(21.22)
Other	45	8.742	(34.97)

*Estimated from actuarial curves.

trial performed on a small number of patients with squamous cell carcinoma [14]. For the same cell types, the median survival was longer than one year in two non-randomized trials using combination chemotherapy and radiotherapy [15, 16].

For other cell types, the comparison of our results with more recent studies is difficult because the group of anaplastic carcinomas included not only small cell but also undifferentiated carcinomas without other specification, and the group "other" is too heterogenous. Recent data seem to indicate a definite advantage of combining different treatment modalities in small cell carcinoma. The median survival with combination chemotherapy plus radiotherapy is longer than 1 yr in a number of recent series, including patients with extensive disease [17–19]. The lack of improvement in survival in the present series with the adjunction of chemotherapy may be due either to the low efficacy of single agent chemotherapy compared to combination chemotherapy in small carcinoma, or to the fact that a possible positive effect in small cell anaplastic carcinoma would have been negated by an adverse effect in other cell types.

The importance of prognostic factors pointed out by others was confirmed in our studies: the prominent value of the performance status as a factor of prognostic significance was stressed by Zelen [20]. Lanzotti *et al.* [21] have found that weight loss was of major importance and is indeed related to anorexia. The same authors mentioned age (>70) as a prognostic factor. This parameter cannot be evaluated in our series because the number of patients older than 70 is quite small, the age limit being 72 in our protocol. The volume of the tumor, its infiltrative character as manifested by laryngeal nerve paralysis and the presence of atelectasis also seem to be important factors.

In conclusion, this trial has not shown a favorable action of long-term chemotherapy with cyclophosphamide in patients with inoperable limited lung cancer treated with radiotherapy but the total dosage of chemotherapy was relatively small. Split-course radiotherapy is as effective as the classical six-week continuous course. Prognostic factors play a very important role in the response of patients to treatment and must be taken into account when preparing protocols of treatment in lung cancer.

STATISTICAL APPENDICES

I. *The Weibull model for survival incorporating covariates*

We assume that the probability of death at time t for the i'th patient is given by

$$f(t)=\lambda_i k t^{k-1}\exp(-\lambda_i t^k) \qquad (1)$$

where $\lambda_i=\underline{\beta}'\underline{\chi}_i$. If there are m covariates then $\underline{\chi}_i$ is an $m+1$ column vector where χ_{i0} is taken as 1 to represent an intercept and the χ_{ij}, $j=1,2,\ldots,m$, represent the values of the m covariates. The $m+1$ column vector $\underline{\beta}$ represents the regression coefficients associated with the covariates and k is a parameter governing the shape of the survival curve. The hazard (instantaneous death rate) for patient i at time i is given by $h(t)=\lambda_i k t^{k-1}$. If $k>1$ the hazard increases with time and the converse is true for $k<1$. For $k=1$ the Weibull model reduces to the simple exponential distribution with a constant hazard. The cumulative survival curve at time t for hazard λ is given by

$$S(t)=1-F(t)=\exp(-\lambda t^k). \qquad (2)$$

The log likelihood for a right-censored sample of size n, $i=1,2,\ldots,n$, is given by

$$\ln L=\sum_{i=1}^{n}[z_i ln(\underline{\beta}'\underline{\chi}_i)+z_i\ln k + z_i(k-1)\ln t_i-(\underline{\beta}'\underline{\chi})t_i^k] \qquad (3)$$

where

$$z_i=\begin{cases}1 & \text{if patient } i \text{ died at time } t_i\\ 0 & \text{if patient } i \text{ was alive at time } t_i\end{cases}$$

The parameters k and $\underline{\beta}$ were estimated by the method of maximum likelihood using an iterative procedure based on a vector analog of the Newton–Raphson procedure.

II. *The logistic regression model for* 0, 1 *response variables*

The logistic model we have used assumes that the relationship between p_i, the probability of response for patient i, $i=1,2,\ldots,n$, and the covariate vector $\underset{\sim}{\chi}_i$ is given by

$$\ln[p_i(1-p_i)]=\underset{\sim}{\beta}'\underset{\sim}{\chi}_i, \tag{4}$$

where for m covariates $\underset{\sim}{\chi}_i$ is an $m+1$ column vector with χ_{i0} taken as 1 to represent an intercept and the χ_{ij}, $j=1,2,\ldots,m$, represent the values of the m covariates. The likelihood for a sample of n observations is given by

$$L=\prod_{i=1}^{n} p_i^{y_i}(1-p_i)^{1-y_i} \tag{5}$$

where $y_i=1$ if observation i was a response and 0 if it was a non-response.

Inverting eq. 4 we obtain

$$p_i=[\exp(\underset{\sim}{\beta}'\underset{\sim}{\chi}_i)]/[1+\exp(\underset{\sim}{\beta}'\underset{\sim}{\chi}_i)]$$

and

$$(1-p_i)=1/[1+\exp(\underset{\sim}{\beta}'\underset{\sim}{\chi}_i)]$$

Using these relationships, taking logarithms and simplifying, we obtain the log-likelihood as

$$L=\sum_{i=1}^{n}[y_i(\underset{\sim}{\beta}'\underset{\sim}{\chi}_i)-\ln(1+\exp(\underset{\sim}{\beta}'\underset{\sim}{\chi}_i)] \tag{6}$$

The vector of regression coefficients $\underset{\sim}{\beta}$ may be estimated by maximum likelihood using an iterative procedure based on a vector analog of the Newton–Raphson procedure.

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An Assessment of the Effects of Steroid Hormones and Antiestrogens on Short-Term Organ Culture of Human Endometrial Carcinoma

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Abstract—*Fifteen human endometrial carcinomas were maintained in organ culture in the presence of various steroid hormones and antiestrogens. The effects of the compounds have been assessed by measuring the incorporation of tritium-labelled thymidine into DNA. No significant effects on tumour cell incorporation rate due to hormones have been observed.*

INTRODUCTION

THE MOST common form of therapy in the treatment of advanced endometrial carcinoma is based upon administration of progesterone or progestational compounds and objective remission of tumours has been observed in approximately one of three patients with pulmonary metastases [1, 2]. The mechanism by which progesterone produces regression of endometrial carcinoma is still uncertain; nor is it known why certain carcinomas respond while others do not. Since hormonal dependence or responsiveness has no specific histological correlation, it would be important if an *in vitro* test to identify hormone-responsive tumours could be devised which might aid in selecting patients for therapy.

Organ cultures have been successfully used by several investigators to study the effects of hormones on both normal and neoplastic tissue growth [3–6]. The advantage of these techniques is that the hormonal effects can be studied directly on individual tissue components without the interference of systemic factors and endocrine feedback mechanisms present *in vivo*. The effect of progesterone on endometrial carcinoma *in vitro* has been studied by Heckmann [7], Hackl [8], and Nordqvist [9, 10]. The latter showed that progesterone acts directly on the cancer cells and that different carcinomas may respond to different concentrations of the hormone. We have conducted a further study of the effects of various steroid hormones and antiestrogens on short term organ culture of human endometrial carcinoma using tritium-labelled thymidine incorporation into DNA to assess therapeutic responses.

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MATERIALS AND METHODS

Culture methods and hormones

A total of 740 explants from 15 endometrial carcinomas have been studied. Specimens were obtained at hysterectomy from patients, aged 46–73, previously untreated for their neoplastic disease. Tissues were placed into sterile containers and transported to the laboratory within 15–30 min. Representative fragments were fixed in 10% formaldehyde and processed for histology. The remainder of the tissue was thoroughly washed with phosphate-buffered-saline (PBS) containing gentamycin (200 μg/ml) and cut under aseptic conditions into small fragments approximately 1 mm thick by 2–3 mm dia. Explants from each tumour were equally divided among different groups (3–9 explants/group, depending upon

the amount of available tissue) and 3 explants were mounted onto a strip of lens paper resting on a stainless grid in a well of a Multi-dish plastic box (Sterilin Ltd, England). The basic culture medium contained Parker TC 199 (Gibco Labs) supplemented with 30% foetal calf serum (Flow Labs), crystalline insulin (5 U/ml), Hepes 20 mM and gentamycin (50 μg/ml). The well was filled with medium in such a way that the surface of the solution was just touching the paper and keeps it moistened. The culture dishes were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed after the first 24 hr of culture and at that time the hormones were added. Hormones were dissolved in ethanol and diluted to a final concentration of 0.1% ethanol in the medium; the same concentration of ethanol was present in control cultures. The hormones used were: progesterone,* 17α-hydroxy-progesterone, Calusterone, Gestonorone capronate, 17β-oestradiol, Nafoxidine, and CI-628.

Assay of tritium-labelled thymidine (^{3}H-TdR) incorporation into DNA

After 3 days in culture from the addition of hormones, tissue explants were transferred to the well of a Multi-dish box containing fresh medium and ^{3}H-TdR (21 Ci/mM, The Radiochemical Center, Amersham) at the final concentration of 0.5 μCi/ml and incubated at 37°C for 60 min. Residues of small uncubation, the explants were thoroughly washed in cold PBS containing 0.1% unlabelled thymidine and lysed in 1 ml of 0.5 M NaOH at 37*C for 60 min. Residues of small undissolved tissue were removed by centrifugation at 3000 rev/min for 10 min. The absorbance at 260 μm of the supernatant was measured. 0.4-ml of 100% trichloroacetic acid (TCA) were added and the tubes were kept in an ice-bath for 30 min. The tubes were centrifuged at 3000 rev/min for 30 min at 2°C and the precipitate was washed three times with 10% TCA. The final precipitate was dissolved in 1 ml of Soluene 350 (Packard Instr.) during 2 hr at 45°C and counted in 10 ml of scintillation fluid (5 g PPO/l. toluene) in a Nuclear Chicago (mod. Marck II) liquid scintillation spectrometer with a counting efficiency of 33%. The results are expressed as counts/min incorporated into TCA-precipitable material ("DNA") per unit absorbance at 260 μm.

*The following common names and abbreviations are used: Calusterone, 17β-hydroxy-7β, 17-dimethyl androst-4-en-3 one (Upjohn); Gestonorone capronate, 17α-hydroxy-19-norpregn-4-en-3,20 dione (Schering); Nafoxidine(U-11, 100A), 1-(-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)]-phenoxy ethyl)pyrrolidine hydrochloride (Upjohn); CI-628, α-(pyrroli-dinoethoxy)-phenyl-4-methoxy-α-nitrostilbene (Parke-Davis).

RESULTS

The tissue showed good preservation of their morphology after 4 days in culture (Fig. 1). To enable meaningful assessment of the effects of hormones, control experiments were carried out to determine the range of ^{3}H-TdR incorporation which could be observed in replicate samples. Seven experiments using separate tumours were conducted using between 6 and 15 replicates in each case. Each replicate sample consisted of 3 explants cultured together in a well of a Multi-dish box as described above. The results are shown in Table 1, which records absolute values and the standard deviations from the mean. The results show a wide scatter and reveal the extent of variation required to be present before definitive hormonal effects can be assessed by the present methods. These wide variations appear to be a reflection of the differing neoplastic cellular components of different areas of the same endometrial carcinoma.

Table 1. Variations in ^{3}H-TdR incorporation between replicate samples in 7 human endometrial carcinomas

Endometrial carcinoma	^{3}H-TdR incorporation* (counts/min/O.D.$_{260\mu m}$)
a	1350 ± 450
b	3225 ± 534
c	4151 ± 2134
d	4241 ± 1087
e	4975 ± 3211
f	6765 ± 2005
g	9989 ± 1555

*Mean absolute values ± S.D.

The incorporation of ^{3}H-TdR in a representative group of tissues cultured with hormones are shown in Tables 2 and 3. Table 2 demonstrates the incorporation in a series of tumours cultured in the presence of progesterone, 17α-hydroxy-progesterone, calusterone capronate, 17β-oestradiol alone or in combination with progesterone, nafoxidine,

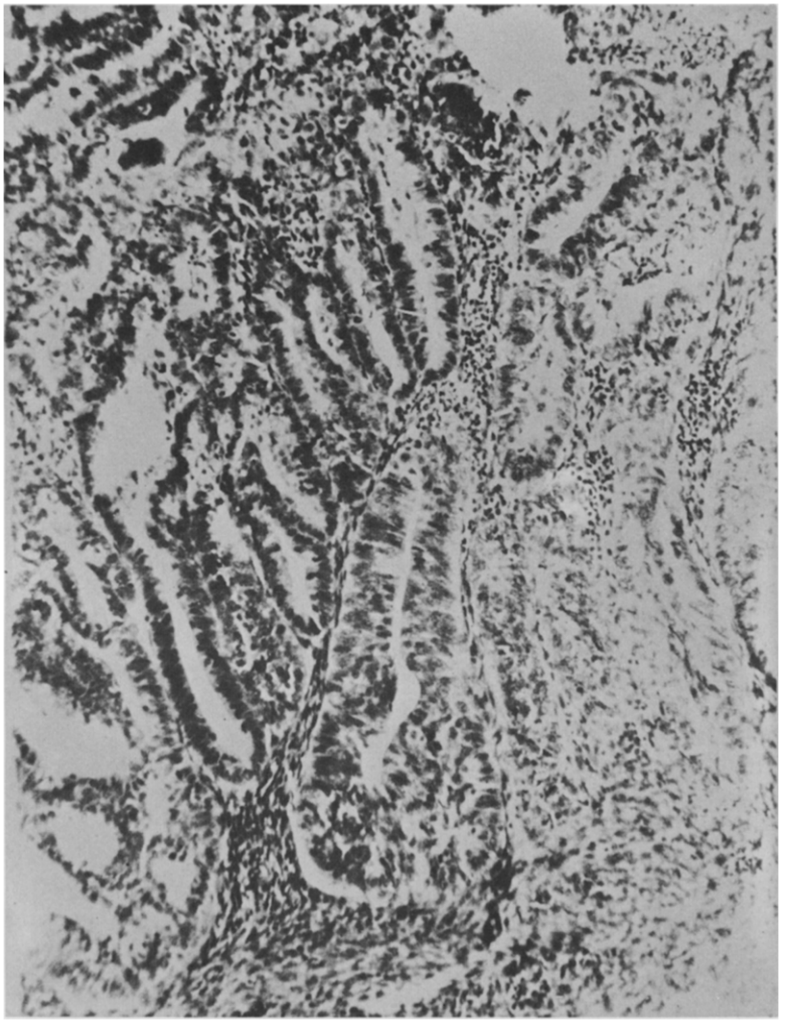

Fig. 1. *Human endometrial carcinoma after culture for* 4 days *in control medium.* (*H and E*, × 250.)

Table 2. ^{3}H-*TdR incorporation in a series of human endometrial carcinomas after* 4 days *in culture*

Experiment No.	^{3}H-TdR incorporation (counts/min/O.D.$_{260\mu m}$) 1	3	4	5	8	11	12
Hormonal treatment							
Control	1146	870	2135	7558	971	4442	1543
Progesterone	764	912	1543	7987	324	3851	1121
17α-hydroxy-prog.	986	234	2987	4321	787	1998	1456
Calusterone	1134	678	1789	2345	1231	3211	974
Gestonorone capr.	667	546	1543	5789	1432	3800	765
17β-oestradiol	1311	987	2001	5800	1106	3987	1127
17β-oestr. + prog.	949	698	2545	8100	761	4654	1287
Nafoxidine	877	265	1981	7002	699	4765	899
CI-628	767	789	1876	5509	901	2987	1345

Explants of human endometrial carcinoma were cultured in the presence of respective hormone at the concentration of 80 μg/ml. After the culture the explants were incubated with ^{3}H-TdR for 90 min as described in Materials and Methods.

Table 3. Effects of different hormone concentrations on incorporation of ^{3}H-*TdR in a series of human endometrial carcinomas*

Experiment No.	^{3}H-TdR incorporation (counts/min/O.D.$_{260\mu m}$) 6	9	10	14
Hormonal treatment				
Control	3456	8977	2898	789
Progesterone (0.8)	3899	7100	2189	1002
Progesterone (8.0)	2567	9100	1543	799
Progesterone (80)	4003	5678	1981	478
17α-hydroxy-prog. (0.8)	2765	6877	3432	698
17α-hydroxy-prog. (8.0)	2001	5878	1999	511
17α-hydroxy-prog. (80)	2200	4398	1876	979
Calusterone (0.8)	5876	6999	3122	199
Calusterone (8.0)	4111	5683	1989	1322
Calusterone (80)	2987	7898	1198	956
Gestonorone capr. (0.8)	1234	4765	1777	976
Gestonorone capr. (8.0)	3111	4444	2198	432
17β-oestradiol (8.0)	4123	7811	1999	961
17β-oestradiol (80)	2981	7555	3111	811
Nafoxidine (8.0)	3997	5899	3221	1087
Nafoxidine (80)	2889	9976	1545	567
CI-628 (8.0)	4000	7012	2341	801
CI-628 (80)	2932	4789	3009	676

Figures in parentheses indicate hormonal concentration in μg/ml.

and CI-628, whilst Table 3 shows the incorporation in the same tumours cultured with varying hormone concentrations. The overall range of ^{3}H-TdR incorporation in these tissues cultured with steroid hormones and antiestrogens is more clearly demonstrated in Fig. 2, which shows the range of incorporation in 740 explants derived from 15 endometrial carcinomas. The tritium incorporation for each sample is expressed as a multiple of the corresponding estimation in a cultured control from the same tumour. Most tissues showed approximately the same level of ^{3}H-TdR incorporation as their cultured control from the same tumours and the majority of the remainder fell within the range which might be expected among replicate samples. One tumour showed a 4-fold decrease in incorporation over its cultured controls in the presence of calusterone; another tumour showed a 6-fold decrease in incorporation in the presence of gestonorone capronate. Finally, 2

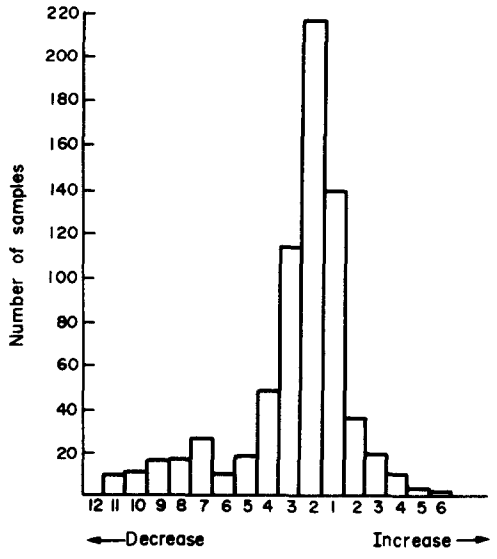

Fig. 2. Tritiated thymidine incorporation in a series of human endometrial carcinomas. The incorporation in each case is expressed as a multiple of that in its cultured control.

tumours showed a 3-fold decrease in incorporation, one in response to nafoxidine and the other to CI-628. 17β-Oestradiol alone or in combination with progesterone did not affect ^{3}H-TdR incorporation.

Applying Friedman's analysis of variance in ranks, we found no group of tumours showing significantly different thymidine incorporation in response to hormones than would be expected in a control group. Similarly, no dose–response effects were observed.

DISCUSSION

Progestagens are the primary choice for therapy of advanced endometrial carcinoma no longer within the reach of surgery or radiotherapy. In a recent study, Reifenstein [11] reported that in 314 women with progressing advanced endometrial cancer and no longer responding to established anticancer measures, therapy with hydroxyprogesterone capronate induced in 37% an objective response consisting of regression in 30.2% and arrest of the disease in 6.8%. Selection of patients likely to respond to hormonal therapy is still largely empirical. Despite extensive research into the endocrine status of individual patients [12], hormone receptor sites concentration [13], and direct effects of hormones on cultured tissue, no predictive tests of clinical value has yet emerged.

The assessment of the effects of hormones on tumours cultured *in vitro* has been most frequently evaluated by organ culture. In general, for solid human tumours, this culture technique can be considered to be close to the *in vivo* situation. One obstacle to the routine use of this technique for an assay system is the great difficulty of assessing the effects of the hormones. Measurement of cell number and viability are often very difficult and most assessments of viability of the tissue after culture have been made on the basis of histological examination of sections of the organ culture, which is extremely difficult to quantitate. The technique of measuring the uptake of a specific DNA label, such as radioactive thymidine, has been successfully used for quantitative measurement of viability of cell in culture [14]. In the case of cultured explants, direct measurement of radioactivity in the tissue seems to be preferable to autoradiographic analysis since radioactive uptake is seen only in the periphery of the specimens, labelling depth does not usually exceed 100–200 μm [15]. Moreover, the distribution of labelled cells often varies considerably in the same tissue, particularly in tumours making determination of labelling index hazardous or even impossible [16].

Using ^{3}H-TdR incorporation into DNA, we failed to demonstrate any significant effect on incorporation due to hormones compared with the controls. These results are in disagreement with those of Hustin [17], who found a significant decrease in incorporation of radioactive thymidine after incubation of endometrial carcinoma with progesterone, even though the method of analysis was by labelling index and not by direct measurement of radioactivity in the tissue and only limited statistical analysis was carried out. His culture conditions are quite similar to those used in our study, the only differences arise from our use of higher concentrations of serum and insulin in the medium. The presence of the last hormone at 5 U/ml resulted in an increase of ^{3}H-TdR incorporation of approximately 30% as compared to culture conditions using insulin at 0.1 U/ml. Moreover, in this study we did not observe the marked fall in incorporation after 3 days of culture which Hustin noted in the absence of hormones and it is possible that optimum *in vitro* growth conditions are not the best for detecting hormonal effects.

The variation in tritium counts between replicate samples indicates that the techniques used here may not be sensitive enough to detect small hormonal variations which might occur in short term organ culture of this particular type of cancer. Recent work by

Gullino *et al.* [18] on hormone dependent tumours in the rat suggests that interpretation of the results from incorporation of tritiated thymidine in terms of tumour growth or regression may not be possible in short term cultures. The variation in experimental results due to the heterogeneity of the tissue poses considerable problems in detecting direct hormonal effects using organ cultures. It is possible that cell monolayer cultures as used by Sekiya *et al.* [19] assessed by incorporation of thymidine into DNA or by survival of cell colonies may eventually yield more satisfactory results for development of *in vitro* tests to predict the therapeutic effects of hormones on endometrial cancer.

Acknowledgements—We thank The Schering Company for supplying Gestonorone capronate, The Upjohn Company for supplying Nafoxidine, and The Parke-Davis Company for supplying CI-628.

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Studies on Informational RNAs and Ribonucleoproteins of Normal Rat Liver, Zajdela Hepatoma and Liver of Hepatoma-bearing Rats

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Abstract—*DNA-like RNA (D-RNA) species, both as constituents of RNP particles and protein-free molecules, isolated from normal rat liver, Zajdela ascites hepatoma and liver of tumor-bearing animals were compared with respect to their sedimentation and chromatographic behaviour. A selective labelling of D-RNA was carried out under actinomycin D-induced block of rRNA synthesis. No detectable differences in nuclear D-RNAs of the tissues compared could be observed whereas cytoplasmic D-RNAs of hepatoma and liver of tumor-bearing rats appear to be much more heterogeneous as compared to those of normal liver. The kinetics of the labelled D-RNAs appearing in the cytoplasm in case of malignant cells also differs from that characteristic of normal liver cells. The comparative analysis of cytoplasmic RNP complexes in the sucrose density and CsCl isopycnic band centrifugation revealed a shift in the ratio of polyribosome-bound and free complexes in favour of the latter in the tumor and liver of tumor-bearing rat as compared with normal liver. The data presented are interpreted to indicate a certain impairment in the selective nucleus-to-cytoplasm transport of RNA in tumor cells, the cells of tumor-bearing animal occupying in this respect an intermediate position between normal and tumor tissues.*

INTRODUCTION

No pronounced differences between nuclear D-RNA species of normal and tumor cells have been revealed so far [1]. Qualitative diversions in the nuclear RNA spectra in neoplasms as compared with homologous normal tissues are relatively small being not constant in their direction in different model systems and presumably can be regarded just as manifestation of tumor progression [2–13], rather than a feature of malignancy itself.

The absence of the above distinctions is surprising since normal *resting* cells have been usually compared with *growing* tumor cells, i.e. the cells in various functional states are used. Indeed, to reproduce itself the tumor cell must synthesize in a short time a great number of different structural and enzymatic proteins including those the normal non-dividing cell does not produce. So, a shift from resting to dividing cells is not necessarily coupled with significant alterations at the transcriptional level as was shown in a number of reports [14–17]. Hence, the important part, which post-transcriptional levels play in the control of protein synthesis in the animal cell, both normal and malignant, may be easily deduced [18–26].

So, it becomes clear that the studies of not only nuclear but also cytoplasmic D-RNAs and RNP complexes are of great importance in comparison of normal and tumor cells.

The paper presented summarizes the data of investigation of sedimentation and chromatographic characteristics of nuclear and cytoplasmic D-RNAs and cytoplasmic RNP complexes containing D-RNAs of normal rat liver cells, Zajdela ascites hepatoma cells as well as of liver of tumor-bearing rats. The latter was meant to reveal possible distant effects of the tumor on the tissues of the host.

MATERIALS AND METHODS

Wistar rats (150–200 g) fasted for 24 hr

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before experimentation were used. One half of a milliliter of a nondiluted suspension of ascites Zajdela cells was transplanted under sterile conditions. The animals were sacrificed 5 days after tumor transplantation. D-RNAs from tissues studied were single (^{14}C) or double (^{3}H and ^{14}C) labelled with 40–100 μCi/rat of radioactive precursors (orotic acid and uridine for liver and hepatoma cells, respectively).

D-RNAs were selectively labelled due to actinomycin D-induced block of rRNA synthesis: 1 hr before sacrifice the animals were injected with 80 μg actinomycin D. In order to obtain a complete block of DNA-dependent RNA synthesis in chase-experiment 1 mg/rat actinomycin D was administered.

In some experiments RNAs of hepatoma cells were labelled *in vitro* by incubation in the Eagle medium, containing 10% bovine serum and 1 μCi/ml ^{3}H-uridine (to inhibit rRNA synthesis the cells were preincubated with 0.05 μg/ml actinomycin D for 30 min).

Nuclear D-RNAs were isolated by means of phenol-temperature fractionation procedure within 50–70°C interval [27]. Cytoplasmic RNA were obtained from the post-mitochondrial supernatant treated with the phenol–chloroform (1:1) mixture in the presence of 0.5% sodium dodecylsulphate.

Cytoplasmic RNP particles were obtained from the post-mitochondrial supernatant by magnesium precipitation according to the method by Leytin and Lerman [28].

RNA fractionation by sucrose density centrifugation and ion-exchange chromatography on the column of methylated albumin-kieselguhr in salt and temperature gradients was conducted as described previously [29, 30].

For a further study of cytoplasmic RNP complexes a novel technique (nucleoprotein-Celite chromatography) elaborated by us recently [31, 32] was used. This method permits fractionation of RNA species according to the tightness of their bonds with proteins within native RNP particles. Cytoplasmic RNAs are separated by this method onto 18S and 28S rRNA, mRNA of polyribosomes and messenger-like RNA of free cytosol particles [32].

Cytoplasmic RNP particles were fractionated by centrifugation in a linear (15–35%) sucrose gradient in a Spinco L2 centrifuge, SW 25.2 rotor at 23000 rev/min and 4°C for 3.5 hr.

CsCl-band centrifugation of RNP particles was performed as described by Spirin *et al.* [33]. RNPs were fixed with formaldehyde for 24 hr (final concn. 4%) before centrifugation on a pre-formed CsCl gradient in a Spinco L2 centrifuge, rotor SW 50, at 44000 rev/min and 4°C for 18–24 hr.

Cytoplasmic poly(A)-containing RNAs were selectively adsorbed on poly(U)-Sepharose [34].

For the determination of radioactivity an RNA carrier (100 μg/ml) and trichloroacetic acid to a final concentration of 5% were added to the samples. In the experiments when a single label was used (only ^{14}C-labelled precursors) the precipitates were collected on Millipore® filters and after drying were measured in scintillation cocktail (4 g of PPO and 0.2 g of POPOP/l. of toluene) in a liquid-scintillation counter Nuclear-Chicago Mark 2 with a counting efficiency of about 70–80% as determined by the channels-ratio method. The samples of RNA double labelled with ^{3}H- and ^{14}C-precursors were collected on Millipore filters as described above followed by the hydrolysis with hot acid (0.5 ml of 1 N HCl per filter at 80°C for 15 min) and counting of the hydrolysate in homogeneous system by mixing the sample with 9 vol of Triton X-100-toluene (1:2) mixture. Discrimination of ^{3}H- and ^{14}C-radioactivity was carried out with the use of external standardization and computation of the results obtained on the "Olivetti Programma 101" system.

RESULTS

Nuclear and cytoplasmic RNAs

Figure 1 represents the methylated albumin-kieselguhr (MAK) chromatography of nuclear RNAs of normal rat liver, liver of tumor-bearing rats, and ascites hepatoma double labelled with ^{3}H and ^{14}C radioactive precursors (2hr and 24 hr, respectively). The MAK-chromatography is known to fractionate RNA molecules according to both their size and nucleotide composition, thus providing the opportunity to study separately ribosomal and informational RNAs [30, 35–37]. Nuclear RNAs are separated by the MAK-chromatography onto two main fractions: the "salt" RNA eluted by NaCl linear (0.6–1.5 M) gradient at 37°C and containing predominantly rRNAs and their precursors and the "temperature" RNA tightly bound to the column and eluted by 1.5 M NaCl and temperature (37°–98°C) gradient (on Fig. 1 both fractions are separated by the arrow). The "temperature"

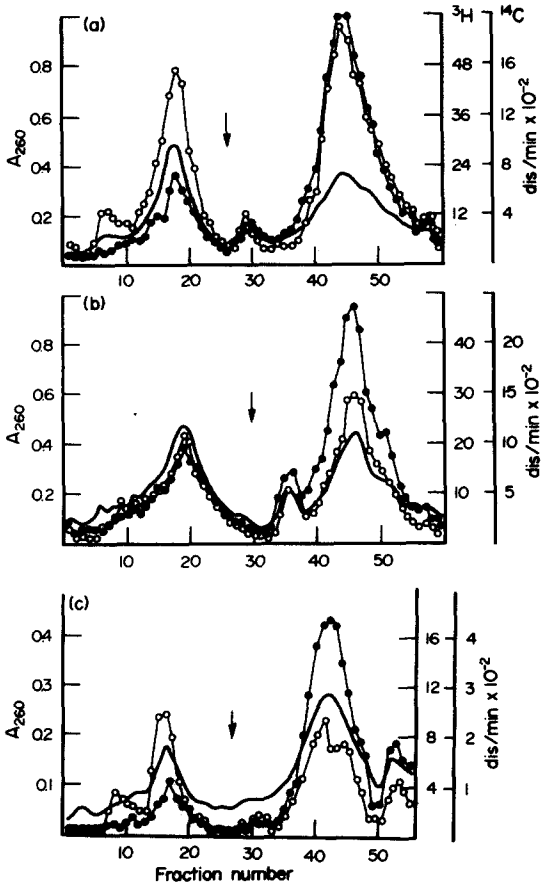

Fig. 1. MAK-chromatography of nuclear RNAs doubled labelled with ^{3}H-orotic acid (100 μCi/rat, 2 hr) and ^{14}C-orotic acid (40 μCi/rat, 24 hr). A—normal liver, B—liver of tumor-bearing rat, C—Zajdela ascites hepatoma (in this case ^{3}H-uridine and ^{14}C-uridine were used). ———, A_{260}; ●——●, ^{3}H-radioactivity; ○——○, ^{14}C-radioactivity. The arrow indicates the start of the temperature gradient.

fraction comprises the bulk of rapidly labelled nuclear heterogeneous RNA [30, 37]. These data taken together with the fact, that "temperature" fraction contains over 70% of nuclear poly(A)-containing RNAs (our unpublished results), suggest that the tenaciously bound fraction represents the nuclear precursors of cytoplasmic mRNAs. However, a certain part of the latter may be localized in the "salt" fraction [36].

The use of a double label showed that the chromatographic distribution of pulse and long-term labelled RNA molecules of the cell nucleus is very similar, though their $^3H/^{14}C$ ratio was found to be different and constantly higher (1.5–3-fold) in the "temperature" fraction as compared with the "salt" one. This fact indicated a more rapid turnover of tenaciously bound RNAs and/or a higher relative content of short-lived molecules among them.

It should be noted here that the chromatographic profiles of the total nuclear RNAs as well as their pulse and long-term labelled subpopulations (the curves of optical density, ^{3}H- and ^{14}C-radioactivity, respectively) were very similar in all three tissues studied.

The MAK-chromatograms of cytoplasmic RNAs labelled under the conditions of actinomycin D-induced block of rRNA synthesis are seen on Fig. 2. In this case the radioactive label is selectively incorporated in 14–16S RNA (presumably mRNA) whereas 18S and 28S rRNAs remain practically unlabelled (not shown here).

The comparison of Figs. 1(a) and 2(a) (the MAK-chromatograms of nuclear and cytoplasmic RNAs from normal rat liver, respectively) shows that in the latter case practically no tenaciously bound "temperature" fraction exists and cytoplasmic mRNAs (radioactivity curve) is localized in the "salt" fraction on the right shoulder of the rRNA peak. Such drastic differences in chromatographic distribution of nuclear pre-mRNAs and their cytoplasmic products reflect apparently the intranuclear processing and selective nucleus-to-cytoplasm transport of RNA molecules.

Unlike nuclear RNAs of all tissues studied whose chromatographic profiles seem to be very close to each other, those of cytoplasmic RNAs are rather different (see Fig. 2). Indeed, proceeding from MAK-chromatograms presented one can state that the

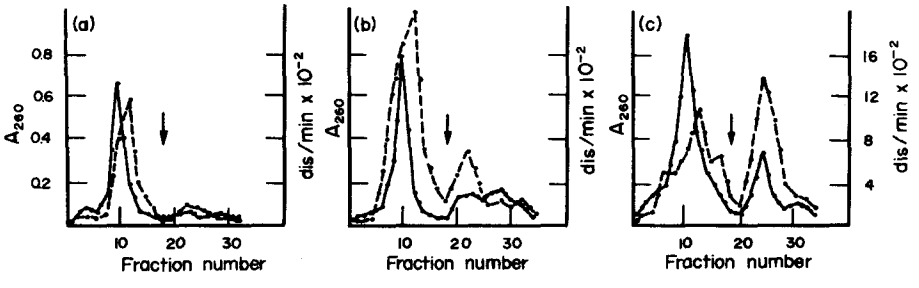

Fig. 2. MAK-chromatography of cytoplasmic RNAs labelled under the condition of actinomycin D-induced block of rRNA synthesis (100 μCi/rat, 60 min). A—normal liver, B—liver of tumor-bearing rat, C—Zajdela ascites hepatoma ———, A_{260}; — —, ^{14}C-radioactivity. The arrow indicates the start of the temperature gradient.

sets of cytoplasmic RNAs from liver of tumor-bearing rat and especially from Zajdela hepatoma cells are more heterogeneous than that from normal liver. In the first two tissues a significant proportion of the rapidly labelled cytoplasmic D-RNA is revealed in the "temperature" fraction which is practically absent from the cytoplasmic RNAs of normal liver cells.

Cytoplasmic poly(A)$^+$-RNAs

A significant proportion of cytoplasmic mRNA is known to be polyadenylated. Figure 3 shows the sedimentation distribution of cytoplasmic poly(A)$^+$-RNA of normal liver cells

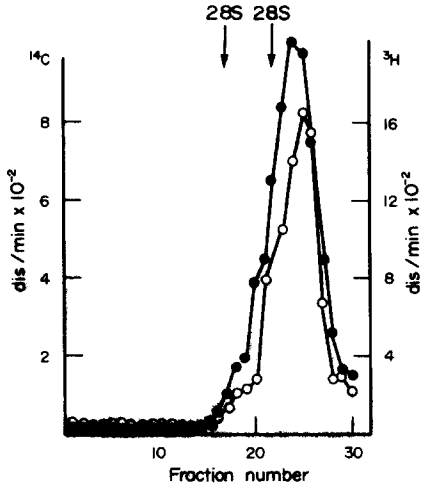

Fig. 3. Sucrose-density-centrifugation of cytoplasmic poly(A)-RNA from rat liver labelled as described in the legend to Fig. 1. ●——●, ^{3}H-radioactivity; ○——○, ^{14}C-radioactivity. The arrows indicate the location of 18S and 28S rRNA markers.

isolated by poly(U)-Sepharose adsorption technique. The RNAs were previously double labelled with ^{3}H- and ^{14}C-orotic acid for 2 hr and 24 hr, respectively. It is seen that the cytoplasmic poly(A)$^+$-RNA sediments as a narrow band with the maximum at 14–16S. It is worth mentioning here that the pulse-labelled poly(A)$^+$-RNAs are somewhat larger in size than the long-term labelled ones as was shown earlier by others [38, 39].

The relative amounts (in %) of poly(A)$^+$-RNA in total cytoplasmic RNA of normal liver, liver of tumor-bearing rat and Zajdela hepatoma before and after complete actinomycin D block of RNA synthesis are presented in Table 1. Two points attract attention. First, the content of poly(A)$^+$-RNA is a little lower in normal liver as compared with that in the two other tissues. Secondly, a complete actinomycin D-induced block of

Table 1. Content of poly(A)-containing RNAs (in % to the total cytoplasmic RNA) in normal liver, liver of tumor-bearing rat and ascites hepatoma Zajdela at different time intervals after actinomycin D-induced block of RNA synthesis (mean values of two independent determinations)

Tissue	Duration of chase (hr)	Content of poly(A)-RNA (%)
1. Normal liver	0	0.92
	2	0.72
	4	0.55
2. Liver of tumor-bearing rat	0	1.03
	2	1.05
	4	1.08
3. Zajdela ascites hepatoma	0	
	2	—
	4	1.17

RNA synthesis entails a rapid reduction of the content of polyadenylated RNAs in normal liver cells but neither in the cells of liver of tumor-bearing animal, nor in the Zajdela hepatoma cells this phenomenon takes place. This fact may indicate a greater stability of polyadenylated RNAs from liver of tumor-bearing animal and hepatoma than those from normal liver. Alternatively, the phenomenon above mentioned may be resulted from a large pool of nuclear pre-mRNAs in the liver of tumor-bearing rat and hepatoma, which is able to maintain a constant nucleus-to-cytoplasm transport of RNA during some time in spite of the arrest of RNA synthesis. From this point of view the normal liver cells contain a relatively small pool of nuclear pre-mRNAs, which is rapidly exhausted after RNA synthesis is blocked.

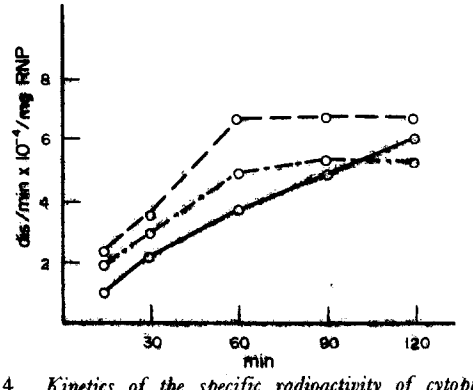

Fig. 4. Kinetics of the specific radioactivity of cytoplasmic RNP particles labelled with 100 μCi/rat of ^{14}C-radioactive precursors during time intervals indicated under the condition of actinomycin D-induced block of rRNA synthesis. ○——○, normal liver, ○—·—○, liver of tumor-bearing rat, ○———○, Zajdela ascites hepatoma.

An indirect evidence favouring the latter possibility was obtained from studying the kinetics of the labelled D-RNA appearing in the cytoplasm of tissues studied (Fig. 4). It could be seen that the cytoplasmic D-RNAs of hepatoma and liver of tumor-bearing rat become labelled very rapidly and reach the plateau earlier than the cytoplasmic D-RNAs of normal liver cells and consequently they turn over rapidly.

Cytoplasmic RNP Particles, containing D-RNA

Centrifugation of RNP particles of tissues studied in sucrose density gradient revealed certain differences in their fractional composition (Fig. 5).

It is seen that in hepatoma cells a relative content of monoribosomes is drastically increased at the expense of polyribosomes—the fact, which is in line with the observations on a number of other tumors [40]. Besides this, in hepatoma cells the content of polyribosomal mRNA (the radioactivity curve) is found to be markedly reduced as compared with rapidly labelled messenger-like RNA of post-ribosomal zone.

As to the sedimentation profile of the RNP particles of liver of tumor-bearing animal,

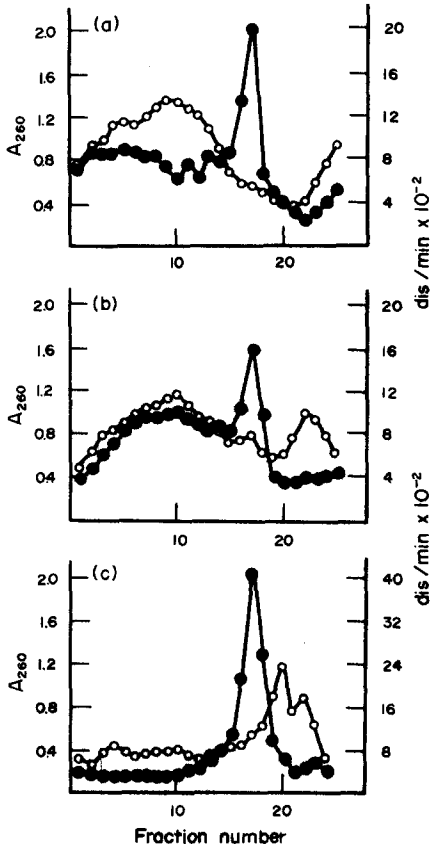

Fig. 5. Sucrose-density-centrifugation of cytoplasmic RNP particles labelled as described in the legend to Fig. 2. A—normal liver, B—liver of tumor-bearing rat, C—Zajdela ascites hepatoma. ●——●, A_{260}; ○——○, ^{14}C-*radioactivity.*

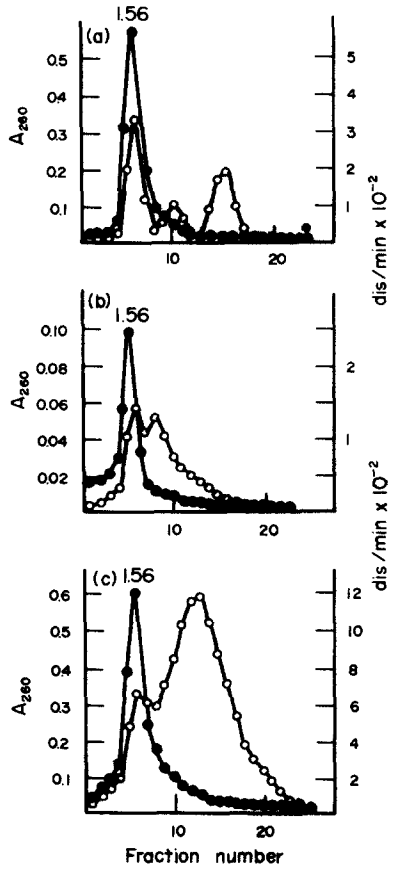

Fig. 6. CsCl-band centrifugation of cytoplasmic RNP particles labelled as described in the legend to Fig. 2 (RNP particles were previously fixed with formaldehyde, see Material and Methods). A—normal liver, B—liver of tumor-bearing rat, C—Zajdela ascites hepatoma. ●——●, A_{260}; ○——○, ^{14}C-*radioactivity. The arrows indicate the location of ribosomes (buoyant density* 1.56 g/cm^3).

it is similar to that of normal tissue, but unlike the latter, the relative content of post-ribosomal messenger-like RNA is somewhat increased.

The above observation of a higher proportion of informosomes in hepatoma and liver of tumor-bearing animals was verified by the results of isopycnic band centrifugation of cytoplasmic RNP particles in CsCl (Fig. 6). This technique permits separation of ribosome-associated mRNP particles (buoyant density 1.56 g/cm^3) from post-ribosomal informosomes (buoyant density 1.38–1.45 g/cm^3). It could be seen from Fig. 6 that in the hepatoma and liver of tumor-bearing rat the proportion of the radioactive material beyond the ribosomes (the main peak of the optical density curve) is significantly higher than that observed in the normal liver.

Recently, a novel fractionation technique (gradient elution of nucleic acids from nucleoprotein-Celite column, NPC-chromatography) was elaborated in our laboratory, which enables a separation of nucleic acids in corres-

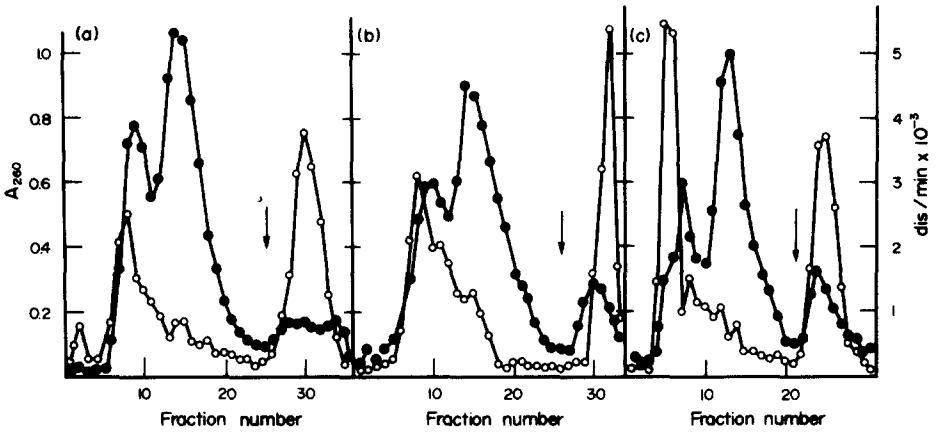

Fig. 7. Nucleoprotein–Celite chromatography of cytoplasmic RNP particles labelled as described in the legend to Fig. 2. A– normal liver, B—liver of tumor-bearing rat, C—Zajdela ascites hepatoma. ●——●, A_{260}; ○——○, ^{14}C-*radioactivity. The arrow indicates the start of the temperature gradient.*

pondence to the tightness of their bonds with proteins within native complexes [31, 32]. This method applied to cytoplasmic RNP particles separates rRNA onto 18S and 28S components (two main components on the optical density curve) and nonribosomal RNAs—onto messenger-like RNA of informosomes and mRNA of polyribosomes (fractions 3–13 and 23–35 on the radioactivity curve of Fig. 7, respectively). The identification of chromatographic components obtained by the nucleoprotein-Celite chromatography was presented earlier [31, 32]. One can see that significant changes in the quantitative proportions of mRNA and messenger-like RNA occur in tissues studied analogous to those obtained by independent methods (see Figs. 5, 6): in liver of the tumor-bearing animal and especially in the hepatoma a proportion of rapidly labelled and non-associated with ribosomes RNAs is greatly increased.

DISCUSSION

The main conclusion which can be drawn from our experiments is that the nuclear DNA-like RNAs of tissues studied are very similar when analysed by the methods used whereas the cytoplasmic nonribosomal RNAs were found to be different in some respects.

The question arises whether differences in cytoplasmic RNAs are due to various extent of nuclear contaminations of preparations studied. Though this possibility can not be completely ruled out at present, it seems very unlikely. First, the kinetics of labelling and actinomycin D-induced decay of nuclear and cytoplasmic DNA-like RNAs were different in all tissues studied (not shown here). Secondly, the cytoplasmic RNP particles of all three tissues appeared to be extremely sensitive to the treatment with EDTA thus demonstrating the characteristic of namely cytoplasmic but not of nuclear RNP complexes.

Finally, a number of current data indicate that the bulk, if not all, of nuclear RNAs are tightly associated with the chromatin fibrils not only at the time of their synthesis but also during all their life-span in the nucleus [41–43]. The appearance of free RNP particles in the nuclear sap and their contamination of cytoplasmic structures thereafter seem to be artifacts caused by the action of endogenous nucleases [21]. In this connection a very low nuclease activity in Zajdela hepatoma cells [44] may be considered as a factor diminishing a nuclear leakage of RNP particles.

On the other hand, the question arises as to the validity of the results obtained with the use of actinomycin D which was shown to exert some side effects including those operating at the level of nucleus-to-cytoplasm RNA transport [45, 46]. However, the doses of the drug which we employed throughout all our work to selectively prevent rRNA synthesis were approximately from one to two order of magnitude lower than those used in "chase" experiments and which appear to interfere with RNA transport. The rates of labelling and the sedimentation velocities of cytoplasmic mRNAs either selectively labelled with the use of actinomycin D or isolated by poly(U)-Sepharose chromatography without treatment with this drug were essentially the same (our unpublished results). Analogous results evidencing against low-dose-actinomycin D-induced damages of mRNA

metabolism were also obtained in our preliminary short-term (less than 30 min) labelling experiments in which rapidly labelled cytoplasmic mRNAs incorporated radioactive precursors selectively since they appeared in the cytoplasm earlier than rRNAs. No notable differences were observed between these mRNAs and those labelled under the conditions of low actinomycin D block with respect to the sedimentation velocities and rate of appearing in the cytoplasm.

As to the "chase" experiments with high doses of actinomycin D which were also carried out in our study (see Table 1) they are more open to criticism. We can not at present rule out the possibility that it was some side toxic effect of actinomycin D that caused observed differences in the rates of a disappearance of cytoplasmic poly(A)-containing RNAs (Table 1), though it seems to us unlikely, especially concerning normal liver and liver of tumor-bearing animals.

With all these reservations in mind and proceeding from the kinetics of labelling and chromatographic profiles of D-RNAs from tissues studied one can suggest the less selectivity of nucleus-to-cytoplasm transport of RNA molecules in hepatoma and liver of tumor-bearing rat as compared with normal liver. A number of observations attest to an important role of the nuclear-cytoplasmic transport in regulation of gene expression in both normal and malignant cells [6, 9, 12, 13, 18–26].

Differences in the tissues studied are manifested also by various distribution of rapidly labelled RNA molecules between polyribosomes and post-ribosomal informosomes, the latter prevailing in liver of tumor-bearing animal and hepatoma unlike the situation in normal liver. The reason for the above phenomenon remains unclear and only speculations on this subject may be put forward. It is possible that there are certain peculiarities in the process of translation in hepatoma cells, e.g. an elevated rate of elongation and termination of polypeptide chains whereas the rate of initiation remains unchanged can result in a decrease in size of polyribosomes and an increase in the pool of monoribosomes and post-ribosomal particles.

According to our observations the liver of tumor-bearing animal occupies an intermediate position between hepatoma and normal liver cells with respect to several properties studied. It seems likely that these alterations are due to distant effects of the tumor on the host's tissues. Recent data of Schumm and Webb are consistent with this proposition [47].

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Circadian Dependence of Host and Tumor Responses to Cyclophosphamide in Mice*

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Abstract—*In a series of five experiments,* 505 *female* BDF_1 *mice were standardized for periodicity studies by exposure to alternating periods of* 12 hr *of light and* 12 hr *of darkness. The mice were inoculated intraperitoneally with* 1 million L1210 *murine leukemia cells and beginning approximately* 24 hr *later, were injected by the same route with a single dose of cyclophosphamide at* 1500, 1800, 2100, 0600 *or* 0900 hr. *At each time point, different groups of mice were treated with* 300 *or* 360 mg *of cyclophosphamide per* kg *of body weight. Cured mice* (60-day *survivors*) *as well as mice dying from drug-induced toxicity or tumor were noted for each interval of treatment.*

The magnitude of the effects produced by cyclophosphamide varied widely from experiment to experiment, suggesting that factors presently unidentified also influenced host responses to the drug. Nevertheless, the composite data indicated that both the therapeutic and the toxic effects of cyclophosphamide were quantitatively related to the circadian phase of drug administration. The frequency of cures was highest (34%) *when the drug was administered at* 1800 hr *and lowest* (7%) *when given at* 0900 hr. *In addition, drug-induced toxicity exhibited a reverse pattern of cyclic activity in which the minimum value* (20%) *resulted from administration at* 1800 hr *while the maximum number of deaths* (52%) *occurred after treatment at* 0900 hr. *Due, in part, to this reciprocal relationship between cure rate and drug-induced death rate, the selective timing of cyclophosphamide administration resulted in greatly divergent modification of the risk versus benefit ratio. As the result of treatment at* 0900 hr, *approximately* 7 *mice were killed for each mouse that was cured while, after treatment at* 1800 hr, 1.7 *mice were cured on the average for each mouse that was killed. Thus by taking advantage of temporal fluctuations in host resistance to cyclophosphamide, enhanced therapeutic responses without equivalent increases in toxicity may be produced.*

INTRODUCTION

THE EFFECTIVENESS of antitumor drugs depends, for the most part, on narrow but exploitable quantitative differences between tumor cell susceptibility and host resistance to chemotherapy [1]. To achieve optimum therapeutic results, therefore, it may be necessary to use dosages that approach the lethal level for the host [2–5]. Thus, any measures that would decrease the toxic component of drug action should improve the prospects for more effective cancer chemotherapy. Studies over the past few years have indicated that toxic as well as beneficial responses to drugs depend significantly on the time of day (or circadian phase) of their administration [6]. Chronotherapy, or the administration of drugs on schedules adjusted to circadian systems, should, therefore, permit the use of higher drug dosages, possibly at more frequent intervals. Evidence supporting this concept and extending it to cancer chemotherapy has been presented [7–16].

Early studies by Badran *et al.* [17] identified the circadian phases during which cyclophosphamide produced maximum and minimum effects on the mitotic activity of a transplantable mammary carcinoma. Subsequently, mitotic rhythms, as well as biochemical and cytokinetic endpoints, served as guides in the design of chronotherapeutic schedules for the administration of cytosine arabinoside, 5-fluorouracil, and actinomycin-D to rats, with emphasis on minimizing host toxicity [7]. These

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studies, which were later confirmed in tumor-free and tumor-bearing mice [8, 13–16], indicated that host toxicity could indeed be reduced through the judicious application of chronotherapy.

Haus *et al.* [15] have shown that the susceptibility of several strains of non-tumor-bearing mice to cyclophosphamide follows a definite circadian pattern. Preliminary results from later studies [10, 12] suggested that, in mice with L1210 leukemia, both tumor and host responses to cyclophosphamide were affected by the time of day that the drug was administered. When different groups of mice were treated at either 1200, 1500, 1800, 2100, 2400, 0300, 0600 and 0900 hr, with varying dosages of cyclophosphamide (180–400 mg/kg), the number of drug-induced, or toxic, deaths was highest among animals treated at 0600 or 0900 hr [10]. The difference in the frequency of toxic deaths between animals at or shortly after the onset of darkness (1800–2100 hr of the day) and those treated at or shortly after the onset of light (0600–0900 hr of the day) was statistically significant. These results allowed us to identify the most appropriate circadian phases (0600–2100 hr) for conducting subsequent studies with cyclophosphamide.

Reported here are results from follow-up experiments that confirm the circadian dependence of cyclophosphamide-induced cures and toxic deaths. The results also support a two-step approach to chronotherapeutic studies: (1) a preliminary identification of circadian phases that include maximum and minimum host sensitivity to the given agent and (2) follow-up studies using this range of time points for final determinations of circadian-dependent responses to the given agent.

MATERIALS AND METHODS

Mice

A total of 505 BDF_1 female mice, obtained either from Simonsen Laboratories, Inc. (Calif.) or Southern Animal Farms (Ala.) through the Division of Cancer Treatment, National Cancer Institute, were used in a series of five experiments conducted over a period of 18 months. When received, the mice ranged in weight from 16 to 21 g. Prior to treatment, they were grouped so that the intercage variation in weight did not exceed 1 g and were exposed to alternating periods of light (0600–1800 hr) and darkness (1800–0600 hr) for at least one week. Temperature in the animal facility was maintained at $23 \pm 2°C$ and the mice were given free access to food (Purina Lab Chow) and water. Between 0900 and 1200 hr of day zero, one day prior to the initiation of treatment, each mouse was inoculated intraperitoneally with one million L1210 murine leukemia cells. In each of the five studies, inoculations were performed only once; therefore, it was possible to inoculate every mouse in a single study with ascites tumor drawn from the same population of tumor cells. In each study, one group of tumor-inoculated mice were injected with 0.9% sodium chloride solution in lieu of cyclophosphamide. The mean post-inoculation life span of these mice served to confirm that the inoculum actually delivered was, indeed, of the intended magnitude. It also afforded one basis for evaluating responses to treatment; nevertheless, toxic deaths and cures were expected to provide more definitive indications of the relative effectiveness of treatment at different times. Thus, the effects produced on these end points by varying the time of drug administration were the chief variables to be assessed. In this respect, the responses manifested by any treatment group served as a partial control for every other group.

Drug treatment

At 1500, 1800 and 2100 hr of the day after tumor transplantation and at 0600 and 0900 hr of the subsequent day, different groups of mice were injected once, intraperitoneally, with cyclophosphamide. At each time, one group of mice was treated with 300 mg/kg while another received 360 mg/kg. Two levels of dosage were used with the expectation that the possibilities for obtaining an equitable distribution of toxic deaths, tumor deaths, and cures would be increased. No significant differences (statistically) were found between these dosages; consequently, in each experiment, the results obtained with 300 and with 360 mg of cyclophosphamide per kg were pooled. All animals were reweighed immediately before drug administration and cyclophosphamide (NSC 26271), supplied by Mead Johnson, was dissolved in 0.9% sodium chloride solution and administered at the rate of 0.01 ml/g of mouse weight.

Collection and evaluation of data

Cures, deaths resulting from drug toxicity or tumor, and the mean survival time of mice dying with tumor were the endpoints by which responses to treatment were gauged. Mice that died before the 10th day after tumor transplantation, without macroscopic evidence of tumor, were considered to have succumbed to

toxic effects produced by cyclophosphamide. Treated animals that survived 60 days after transplantation and, at that time, lacked gross evidence of tumor, were considered cured. From the collected data, estimates of central value and dispersion were calculated for the various treatment groups. The statistical inference of inter-group responses to cyclophosphamide was evaluated by Chi-square analysis and by analysis of variance (ANOVA).

RESULTS

Collectively, in five replications of the same experiment, cyclophosphamide was administered once to each of 480 tumor-bearing mice. The results, in terms of cured mice and those mice that were killed by the tumor or by the drug, itself, are presented in Table 1.

Tumor deaths

Altogether, 202 mice, 42% of those treated, died from effects produced by the tumor. Statistical ANOVA indicated that both the frequency of tumor-induced deaths and the post-inoculation life span of mice killed by the tumor were independent of the photo-periodic timing of drug administration. The incidence with which treated mice were killed by tumor varied significantly among the different replicates of the study ($P>0.01$) but the post-inoculation life span of such mice did not differ significantly as a function of replication. Those 202 treated mice lived 23.9 ± 7.8 days on the average;

Table 1. *Responses of tumor-bearing mice to cyclophosphamide administered at different times of day*

Exp. No.	End points evaluated	Treatment time (hr of the day) 1500	1800	2100	0600	0900	Total Experiment
Cures*		4/20	9/20	3/20	1/20	0/20	17/100
1	Toxic deaths*	7/20	0/20	5/20	16/20	20/20	48/100
	Tumor deaths*	9/20	11/20	12/20	3/20	0/20	35/100
	(MST ± 1SD)†	(26.3 ± 10.5)	(22.1 ± 2.8)	(22.3 ± 4.2)	(21.7 ± 0.6)		(23.2 ± 6.1)
	Cures	0/20	4/20	3/20	3/20	1/20	11/100
2	Toxic deaths	19/20	13/20	14/20	15/20	19/20	80/100
	Tumor deaths	1/20	3/20	3/20	2/20	0/20	9/100
		(25.0 ± 0.0)	(42.0 ± 17)	(36.7 ± 21.1)	(16.5 ± 2.1)		(32.7 ± 17.2)
	Cures	9/20	5/20	8/20	5/20	1/20	28/100
3	Toxic deaths	3/20	1/20	6/20	4/20	1/20	15/100
	Tumor deaths	8/20	14/20	6/20	11/20	18/20	57/100
		(21.8 ± 4.6)	(25.4 ± 9.7)	(20.0 ± 3.6)	(22.9 ± 5.8)	(22.2 ± 6.3)	(22.8 ± 6.8)
	Cures	4/18	5/18	2/18	2/18	2/18	15/90
4	Toxic deaths	4/18	4/18	3/18	4/18	8/18	23/90
	Tumor deaths	10/18	9/18	13/18	12/18	13/18	52/90
		(24.8 ± 4.9)	(20.4 ± 4.9)	(22.6 ± 4.7)	(29.9 ± 11.2)	(20.0 ± 4.6)	(23.9 ± 7.5)
	Cures	9/18	10/18	6/18	6/18	3/18	34/90
5	Toxic deaths	3/18	1/18	1/18	0/18	2/18	7/90
	Tumor deaths	6/18	7/18	11/18	12/18	13/18	49/90
		(24.5 ± 2.7)	(23.7 ± 7.6)	(26.6 ± 5.7)	(25.1 ± 10.6)	(22.2 ± 4.2)	(24.4 ± 6.9)
	Cures	26/96	33/96	22/96	17/96	7/96	105/480 (22%)
Totals‡	Toxic deaths	36/96	19/96	29/96	39/96	50/96	173/480 (36%)
	Tumor deaths	34/96	44/96	45/96	40/96	39/96	202/480 (42%)
		(24.4 ± 6.4)	(24.4 ± 9.1)	(24.1 ± 7.5)	(25.3 ± 9.4)	(21.7 ± 5.3)	(23.9 ± 7.8)

*Expressed as number of mice per treatment group.

†Mean survival time ± 1 S.D. post inoculation for mice that died with tumor. Tumor bearing mice not treated with cyclophosphamide served as controls in each experiment. The mean day of death for controls (pooled data) was 7.1 ± 1.1 days after tumor inoculation.

‡The difference between cures at 0900 and 1800 hr was highly significant by Chi-square analysis ($P<0.01$), as was the difference between toxic deaths at these time points ($P<0.005$). See text for comparisons between other groups. The percentages of mice cured or dying from drug toxicity, as function of time of treatment are shown in Fig. 1.

thereby, they exceeded the life span of 23 untreated, tumor-inoculated mice (7.1 ± 1.1 days) by 16.8 days (237%).

Cured mice

Twenty-two per cent (105) of the mice that were injected with cyclophosphamide were cured. According to ANOVA, the frequency with which cures were produced varied significantly with replication ($P<0.1$) and also with the timing of drug administration ($P<0.01$). The maximum cure rate, 34%, was produced when cyclophosphamide was given at 1800 hr. By Chi-square evaluation, it exceeded significantly that obtained when the drug was given either at 0600 hr or at the nadir of response, 0900 hr ($P<0.01$ at least). Furthermore, the cure rates produced by treatment at 1500 hr and at 2100 hr also exceeded, significantly, the 7% rate of cure produced by treatment at 0900 hr.

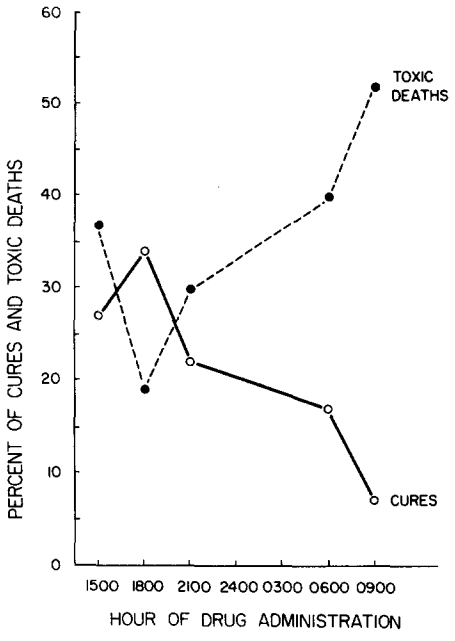

Fig. 1.

Drug-induced lethality

Of the 480 mice that were treated with cyclophosphamide, 173 (36%) died from toxic effects that were produced by the drug. By ANOVA, the frequency with which mice were killed by cyclophosphamide varied significantly with replication ($P<0.01$) but was indicated to be independent of the timing of drug administration. Substantial differences in toxicity were noted, however, that, nevertheless, appeared to be correlated with the timing of treatment. In terms of drug-induced lethality, maximum toxicity was produced by treatment at 0900 hr: 52% (50 of 96) of the mice treated at that time were killed by the drug. Conversely, only 20% (19 of 96 mice) were killed by treatment at 1800 hr. By Chi-square analysis, the frequency of toxic deaths after treatment at 0900 hr was significantly greater than that produced by treatment either at 1800 hr or at 2100 hr ($P<0.005$). In addition, toxic deaths also occurred in significantly greater numbers when cyclophosphamide was given at 0600 hr than after treatment at 1800 hr.

DISCUSSION

The composite data from the five experiments that have been described indicate that the therapeutic as well as the toxic effects produced by cyclophosphamide were clearly related quantitatively to circadian systems of the host. Significantly more mice were cured when cyclophosphamide was administered at 1800 hr than when given at 0600 or 0900 hr. Even when the drug was given at 1500 or 2100 hr, times that were, respectively, 3 hr earlier and 3 hr later than the optimal time of 1800 hr, the cure rates that were produced significantly exceeded the 7% nadir that resulted from treatment at 0900 hr. On the other hand, for reasons which are unclear at the present time, neither the frequency of tumor-induced deaths nor the post-inoculation life span of the mice that died with tumor were altered significantly by varying the time of treatment.

As depicted in Fig. 1, the order of drug-induced deaths was essentially the reverse of the pattern with which cures were produced. Maximum toxicity was produced by treatment at 0900 hr, the nadir for cure rate. Conversely, the nadir for toxic deaths was produced by treatment at 1800 hr, the time that resulted in the maximum rate of cure.Because of this reciprocal relationship between cure rate and drug-induced death rate, the overall therapeutic advantage derived from the selective timing of drug administration appears to have been compounded. Not only were fewer mice killed, an improvement in itself, but, in addition, some of the extra mice that survived the effects of the drug were also cured. The risk versus benefit relationship is presented in Table 2 in which the ratio of cured mice to mice killed by drug is recorded. At 0900 hr, the time at which treatment proved most toxic and produced the fewest cures, approximately 7 mice were killed by treatment for each mouse cured. On the other hand, when cyclophosphamide was administered at 1800 hr, 1.74 mice were cured on the average for each mouse that was killed by the drug.

Table 2. Risk versus benefit ratios observed with the administration of cyclophosphamide at different times of the day

Time of treatment*	No. cures	No. toxic deaths	Risk vs benefit ratios: Cures/toxic deaths	Risk vs benefit ratios: Toxic deaths/cures
1500	26	36	0.72	1.39
1800	33	19	1.74	0.58
2100	22	29	0.76	1.32
0600	17	39	0.44	2.29
0900	7	50	0.14	7.14

*A total of 96 BDF_1 mice bearing L1210 leukemia were treated at each treatment time (hr of the day).

The susceptibility of tumor-free mice to treatment with cyclophosphamide has been also reported to be circadian dependent [15]. In addition, these workers noted pronounced variation in such rhythmicity among 5 studies in which differences in mouse strain and sex constituted uncontrolled experimental variable of unknown importance.

In the studies described in the present report, L1210-inoculated, BDF_1, females were used exclusively. Nevertheless, only the post-inoculation life span of treated mice killed by the tumor was independent of the variability produced by replication. Statistically significant inter-replicate variation was identified with respect to the frequency of drug-induced deaths, the cure rate, and the incidence with which deaths were produced by the tumor. Such inter-experimental variability suggests that factors presently unidentified can also influence host responses to cyclophosphamide.

Thus, while unidentified variables continue to limit its full potential, the value of chronotherapy in the management of neoplasia has been established and, certainly, additional studies should be conducted to identify the circadian phases of susceptibility for those other agents that are currently used alone and in combination in experimental and clinical chemotherapy.

List of compounds

Cyclophosphamide: NCS-26271; CAS Reg. No. 6055-19-2; 2H-1,3,2-oxaza-phosphorize, 2 (bis(2-chloroethyl)amino) tetrahydrozoxide, monohydrate.

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Serological Reactivities of Human and Baboon Sera Against EBNA and *Herpesvirus papio*-determined Nuclear Antigen (HUPNA)*

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Abstract—*Using our recently developed acid-fixed nuclear binding technique, an EBNA-like antigen was detected in both virus-producer and non-producer baboon lymphoid cell lines that carried* Herpesvirus papio (*HVP*). *We designated the antigen as HUPNA. Serological comparison of EBNA and HUPNA showed that:* (i) *all EBNA-positive sera reacted with both EBNA and HUPNA;* (ii) *none of the anti-HVP(VCA)-positive baboon sera reacted with EBNA; and* (iii) *approximately half of the anti-HPV(VCA)-positive baboon sera tested reacted with HUPNA. HUPNA could not be visualized by anticomplement immunofluorescence* in situ.

INTRODUCTION

Herpesvirus papio (HVP) is an Epstein–Barr virus (EBV)-related lymphotropic herpesvirus of baboons [1, 2]. HVP-producer lines express early antigen (EA) and viral capsid antigen (VCA), readily detected with human sera containing antibodies to EBV [1]. However, neither HVP-producer nor non-producer lines have been found to express a nuclear antigen by anticomplement immunofluorescence (ACIF) staining *in situ*. In a previous paper [3], we reported the presence of an EBNA-like nuclear antigen in HVP-transformed lymphoid lines, using our recently developed acid-fixed nuclear binding (AFNB) technique [4]. AFNB provides an amplification system for antigen detection, compared to ACIF staining *in situ*. We have found that HUPNA could be detected with EBNA-positive human sera [3]. It was previously shown, however, that anti-HVP(VCA)-positive baboon sera do not react with EBNA. It was therefore of interest to explore whether any of the baboon sera contain antibody against HUPNA, as detected by the AFNB technique. In this paper, we show that this is indeed the case and some but not all baboon sera contain anti-HUPNA antibodies that do not react with human EBNA. We are thus dealing with a certain "asymmetrical" cross reactivity. A somewhat similar situation was found previously by Gerber *et al.* [5, 6] in relation to the EBV-related chimpanzee virus (*Herpesvirus pan*), detectable by ACIF staining *in situ*.

MATERIALS AND METHODS

The baboon (*P. hamadryas*) HVP-DNA-carrying non-producer line 26CB-1 [1] and the EBV-carrying human non-producer lymphoma line Raji [7, 8] were used to prepare complement-fixing (CF) soluble antigens. An EBV-negative human lymphoma line Ramos [9], a marmoset fibroblast line HF-2 [10], and chicken red blood cells (CRBC) were prepared as targets for the AFNB technique. All lines were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 100 i.u. penicillin and 100 μg streptomycin per ml. Preparations of EBNA and HUPNA, acid-fixed nuclear binding to

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methanol–acetic acid-fixed targets and ACIF staining were performed as previously [3, 4, 11]. *In situ* ACIF tests were carried out on methanol-acetone (1:2)-fixed cells [12].

RESULTS AND DISCUSSION

The sera used in this study were: Human—anti-EBV(VCA)+/anti-EA- (healthy donors, anti-EBNA 1:80), anti-EBV(VCA)+/anti-EA+ (African Burkitt lymphoma patients, anti-EBNA 1:80—1:640), or no antibodies to EBV (controls, anti-EBNA<1:2); Baboon—anti-HVP(VCA)+/anti-EA−; Squirrel monkey—anti-HVP(VCA)-/anti-EA− (Table 1).

In the ACIF test for EBNA, EBNA-positive human sera stained both Raji and AW-Ramos cells [9] but failed to detect any EBNA-like antigens in baboon cells (8CB-1, 13CB-1 and 26CB-1) [1]. Anti-VCA-positive baboon sera did not stain EBNA in Raji or in AW-Ramos cells and detected no EBNA-type antigens in HVP-carrying baboon cells. The AFNB technique detected the HVP-associated nuclear antigen (HUPNA) in a HVP-DNA-carrying non-producer baboon line (26CB-1) and also in producer lines [3]. As shown in Table 1, all EBNA-positive human sera reacted with both EBNA and HUPNA, bound to three different target cells (CRBC, Ramos and HF-2). No positive ACIF-reactions were obtained with anti-EBNA-negative human sera. Using HF-2 cells as targets, approximately half of HVP(VCA)-positive baboon sera tested stained HUPNA (7/16), although they did not react with EBNA. Neither EBNA nor HUPNA was stained by HVP-negative squirrel monkey

Table 1. Detection of EBNA and HUPNA by the AFNB technique

Sera	ACIF-reactions for EBNA* binding to			HUPNA† binding to		
	CRBC	Ramos	HF-2	CRBC	Ramos	HF-2
Human EBV+	8/8‡	5/5	4/4	7/7	5/5	4/4
EBV−	0/4	0/3	0/3	0/3	0/3	0/2
Baboon HVP+						
D26	...§	...	...	...	...	−
D30	...	−	...	...	+	+
807	...	...	...	...	...	−
1830	...	...	...	...	...	−
1935	...	...	...	...	...	−
2227	...	...	...	...	...	−
2248	...	...	...	...	...	−
3016	−	−	−	...	+	+
3017	...	...	...	...	...	−
3019	...	...	...	...	...	−
3021	−	−	−	+	+	+
3048	−	−	−	...	+	+
pool 1	−	−	−	+	+	+
pool 2	−	−	−	+	+	+
pool 3	−	−	−	+	+	+
Russian baboon	...	...	...	...	−	−
All baboons	0/6	0/7	0/6	4/4	7/8	7/16
Squirrel monkey HVP−						
2209	...	−	...	...	−	−
2210	−	−	−	−	−	−
4056	...	−	...	...	−	−
All squirrel monkeys	0/1	0/3	0/1	0/1	0/3	0/3

*EBNA: CF-antigen from Raji cells. †HUPNA: CF-antigen from 26CB-1 cells. ‡Number sera positive/number sera tested; sera tested at 1:10 for human and 1:4 for baboon and squirrel monkey. §Not tested.

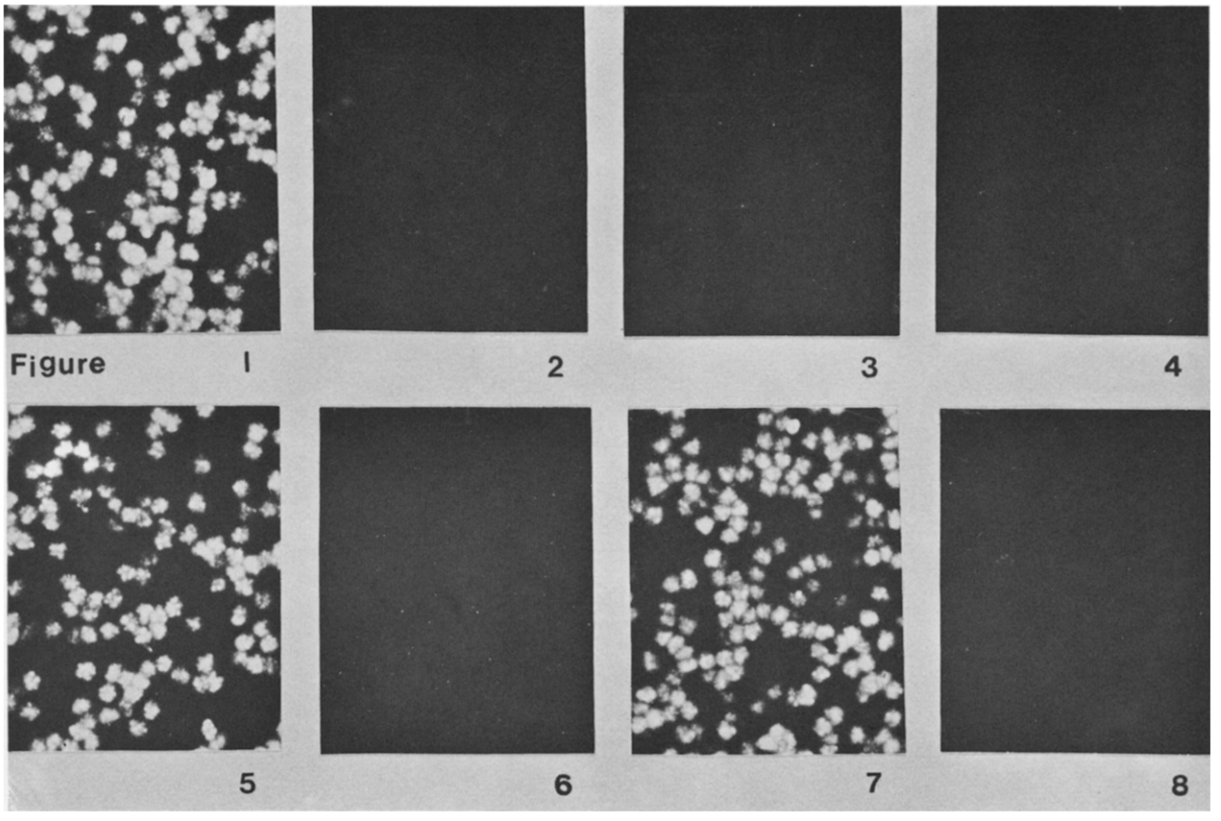

Fig. 1. *Methanol–acetic acid-fixed CRBS were incubated with Raji-CF, followed by anti-EBNA-positive human serum. Positive fluorescence for EBNA was seen.* × 680.

Fig. 2. *Negative EBNA staining. Same as Fig.* 1, *with anti-EBNA-negative human serum.* × 680.

Fig. 3. *Same as Fig.* 2, *with HVP-positive baboon serum.* × 680.

Fig. 4. *Same as Fig.* 2, *with HVP-negative squirrel monkey serum.* × 680.

Fig. 5. *Same as Fig.* 1, *except that the fixed CRBC were incubated with* 26*CB*-1-*CF and anti-EBNA-positive human serum. Positive fluorescence for HUPNA was seen.* × 680.

Fig. 6. *Negative HUPNA staining. Same as Fig.* 5, *with anti-EBNA-negative human serum.* × 680.

Fig. 7. *Same as Fig.* 5, *with HVP-positive baboon serum. Positive fluorescence was seen.* × 680.

Fig. 8. *Same as Fig.* 6, *with HVP-negative squirrel monkey serum.* × 680.

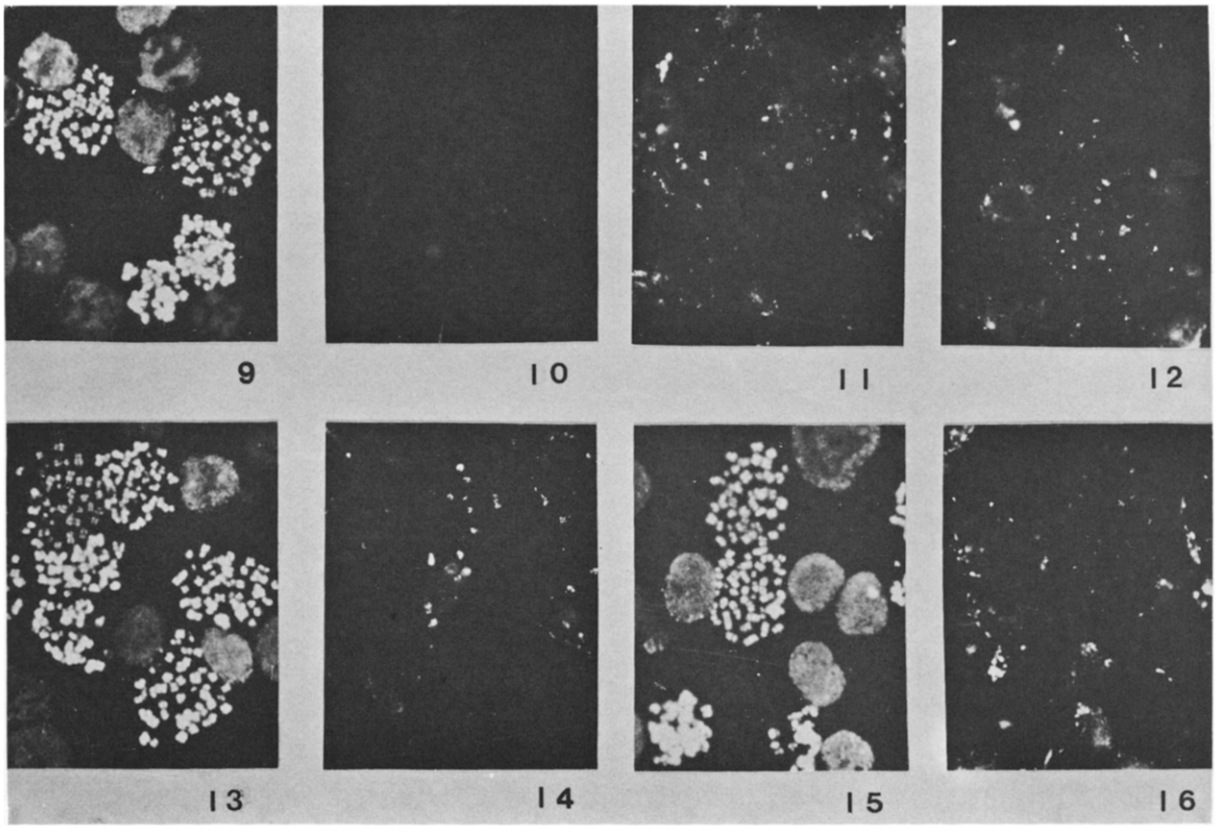

Fig. 9. *Methanol–acetic acid-fixed HF-2 cells were incubated with Raji-CF, followed by anti-EBNA-positive human serum. Both interphase nuclei and metaphase chromosomes were positively stained.* × 680.

Fig. 10. *Negative EBNA staining. Same as Fig.* 9. *with anti-EBNA-negative human serum.* × 680.

Fig. 11. *Same as Fig.* 10, *with HVP-positive baboon serum.* × 680.

Fig. 12. *Same as Fig.* 10, *with HVP-negative squirrel monkey serum.* × 680.

Fig. 13. *Same as Fig.* 9, *except that the fixed HF-2 cells were incubated with 26CB-1-CF and anti-EBNA-positive human serum. Positive fluorescence for HUPNA was seen on both interphase nuclei and metaphase chromosomes.* × 680.

Fig. 14. *Negative HUPNA staining. Same as Fig.* 13, *with anti-EBNA-negative human serum.* × 680.

Fig. 15. *Same as Fig.* 13, *with HVP-positive baboon serum. Positive fluorescence for HUPNA was seen.* × 680.

Fig. 16. *Same as Fig.* 14, *with HVP-negative squirrel monkey serum.* × 680.

sera. When CRBC were used as targets for CF-antigen binding, all sera from baboon and squirrel monkey were absorbed with acid-fixed CRBC prior to the AFNB test because of nonspecific anti-CRBC reactions with the sera.

The staining reaction obtained by the AFNB procedure is illustrated by Figs. 1–16. The positive ACIF-reactions were shown in Fig. 1 (CRBC/EBNA/human serum), Fig. 5 (CRBC/HUPNA/human serum), Fig. 7 (CRBC/HUPNA/baboon serum), Fig. 9 (HF-2/EBNA/human serum), Fig. 13 (HF-2/HUPNA/human serum) and Fig. 15 (HF-2/HUPNA/baboon serum). Figs. 2, 3, 4, 6, 8, 10, 11, 12, 14 and 16 show negative controls. In HF-2 cells (Figs. 9, 13 and 15), ACIF-reactions were positive not only with interphase nuclei but also with metaphase chromosomes.

These experiments revealed that: (i) EBNA-positive human sera reacted with both EBNA and HUPNA, (ii) none of baboon sera reacted with EBNA and (iii) approximately half of baboon sera (7/16) gave a positive staining with HUPNA. These results indicate that EBV-positive human sera contain antibodies that react with both EBNA and HUPNA while HVP(VCA)-positive baboon sera either contain no detectable antibodies to EBNA or HUPNA, or react with HUPNA but not EBNA.

The presence of an EBNA-like nuclear antigen was recently demonstrated in chimpanzee lymphoid lines [5, 6]. Chimpanzee virus-carrying lymphoid lines contain DNA sequences homologous to approximately 34–45% of human EBV–DNA [5]. The antigen (Ch-NA) was stained by some human, chimpanzee or orangutan sera in ordinary ACIF.

Different explanations may be considered for the asymmetrical cross reactivity between EBNA and HUPNA. By and large, human anti-EBNA-positive sera have higher titers against a variety of EBV-related antigens, compared to the corresponding reactivity of the baboon sera against baboon HVP-related antigens. HUPNA is present in lower concentration than EBNA, because it can not be detected by ACIF staining *in situ*. Therefore, anti-EBNA-positive human sera are more comparable to hyperimmune sera than anti-HUPNA baboon sera that may reflect a less intense or less prolonged immunization. It is known that hyperimmunization tends to induce more cross reactive antibodies than immunization at a less intense or less prolonged level. Alternatively, the asymmetrical cross reactivity may reflect a true antigenic difference, rather than the different consequences of hyper vs low degree immunization. If so, it would follow that human EBNA has additional specificities, over and beyond the specificities that cross react in HUPNA and EBNA. These specificities would only be detected by the human anti-EBNA-positive, but not the baboon anti-HUPNA-positive sera. Absorption experiments should clarify this question.

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Polycyclic Hydrocarbon-Induced Rat Sarcomas Correlated to Disturbances in the Deoxyadenylate Regions of the Tumor DNAs*

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Abstract—*Ten separate groups containing a total of* 51 *rat sarcomas induced by the polycyclic hydrocarbons were examined for inducing agent-related abnormalities in their DNAs. The levels of deoxyadenylate (dA) regions in normal and tumor rat DNAs were estimated from annealing* [^{3}H]*-polyuridylic acid to DNA and treatment of the resultant hybrids with ribonuclease A. All the tumor material had significant reductions in the (dA) regions of their DNAs when the levels were compared to normal rat DNA. Although the (dA) reductions were rather heterogeneous from one tumor group to the other no matter the inducing agent, the greatest decreases tended to be found with tumors induced by* 20*-methylcholanthrene (MC) and* 7,12*-dimethylbenz(a)anthracene (DMBA). No relationship was found between (dA) alterations and the types of chromosomal aberrations in the tumor cell populations. However, normal and tumor DNAs, fractionated into A+T rich and G+C rich DNA by thermal elution from hydroxylapatite columns, showed specific neoplastic-associated (dA) disturbances in the A+T rich DNA of tumors induced by* 3,4*-benzopyrene (BP), MC and DMBA. The base compositions of the tumor material were also analyzed and compared to normal rat DNA. Tumors induced by DMBA showed highly significant decreases in* % *A+T whereas BP and MC induced tumors tended to show only small increases in* % *A+T. Some tumors induced by BP, MC and DMBA were unaltered in base composition. Together our results are consistent with the hypothesis that DMBA is the strongest carcinogen in the series because it interacts preferentially with A+T rich DNA, which is the type of DNA most important for polycyclic hydrocarbon-induced neoplasm in the rat.*

INTRODUCTION

THE PRESENCE of (dA) rich stretches and polypyrimidine runs in eukaryotic DNA has now been validated by several laboratories [1–11]. The (dA) or (dT) rich sequences and other polypyrimidine stretches were approximately 25–200 nucleotides in length [2–4, 6, 10] and were concentrated into repeated sequences in the DNA [4, 7, 11]. The fact that these oligohomopolymeric rich regions could not have arisen from a random selection model [1], together with their specificity for location into repeated DNA, have strongly suggested a biological function for these regions.

These observations are further supported from similar work on nRNA. It has been shown that oligo(U) and oligo(A) regions of approximately 25–35 nucleotides are a normal component of nRNA [12–14] indicating that their template for transcription must have been the (dA) or (dT) regions in the DNA [13]. The oligo (U) regions in nRNA have also been shown to be located primarily in the

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The abbreviations used are: (dA), deoxyadenylate; (dT), deoxythymidylate; (U), ribouridylate; (A), riboadenylate; BP, 3,4-benzopyrene; MC, 20-methylcholanthrene, DMBA, 7,12-dimethylbenz(a)anthracene; SSC, 0.15 M NaCl + 0.015 M sodium citrate, pH = 7.2; T_M, temperature at which 50% native DNA becomes single stranded nRNA, nuclear RNA.

5'-end of nRNA which is mainly transcribed from repeated DNA template [14, 15] and confirms the location of (dA) clusters there. Since the 3'-end of nRNA contains unique DNA transcript or the mRNA, then the implications are that (dA) transcripts or the oligo(U) regions might play a role in the proposed processing of nRNA into mRNA [16]. At any rate, the exact function of (dA) transcripts is still unknown although some biological function most certainly can be implied.

Our laboratory has assumed an important function for the (dA) regions in either the transcription or processing of genetic information, and has attempted to assign a role to them in neoplasm. Our results have shown that (dA) alterations could be demonstrated in the DNA from human breast tumors [17], human CLL lymphocytes [18] and rat tumors induced by Rous sarcoma virus or DMBA [19]. Generally, the more malignant cell types were associated with reduced levels of (dA) regions in their DNAs. Although (dA) disturbances were found in both A+T rich and G+C rich DNA [17], some specificity of change was observed for both main band and low repeated DNAs (18). However, our ability to demonstrate specific (dA) alterations common to neoplasm has been complicated by looking at neoplasms where the oncogenic agent is not known. Presumably, the (dA) alterations caused from viral carcinogenesis where the viral genome is involved could be quite different from those arising from chemical carcinogens. Therefore, in this study we have examined rat tumors induced by a class of closely related carcinogens, the polycyclic hydrocarbons, and where the carcinogenic potency varies in a predicted way [20, 21]. With this well defined tumor induction model, we have tried to show that tumors induced by structurally-related chemicals have (dA) alterations in specific areas of their DNA.

MATERIALS AND METHODS

Tumor induction and normal tissue

Inbred Wistar–Furth rats 8–35 days of age were inoculated subcutaneously in the right thigh with 1–4 mg doses of BP, MC or DMBA dissolved in 0.1 ml tricaprylin. Tumors developed to 100% and were harvested 6–8 weeks after they were palpable (<7 months). Some individual tumors were analyzed for karyotypic abnormalities by direct fixation according to Levan *et al.* [22]. Other tumors induced by the same carcinogen were pooled and not karyotyped. The groups of tumors designated DMBA-17 and DMBA-19 were transplanted for a single passage in newborn Wistar–Furth rats as described elsewhere [23]. All tumors were frozen and stored at −70°C until used for DNA extraction.

The livers, spleens, inner skin covering the abdominal cavity and full term embryos were dissected from adult Wistar–Furth rats and either processed directly or stored at −70°C. These tissues were taken from apparently healthy animals and served as the control sources of normal rat DNA.

Extraction of DNA

The rat tissues were minced in ice cold 0.1 M EDTA–0.1 M NaCl, pH 7.5 and homogenized at 4°C by 2 × 15-sec pulses at 1 min intervals with an Ultra-Turrax. The DNA was extracted from the homogenates by the Marmur method [24] except for the final alcohol precipitation. Instead of the precipitation step, 100 μg/ml ribonuclease A (Sigma) and 100 μg/ml alpha amylase (Sigma) were added to the DNA solutions and the mixture dialyzed overnight against 5 l 0.1 × SSC at room temperature. Finally, the enzyme-treated DNA solutions were made 5% sodium dodecyl sulfate and precipitated with alcoholic sodium perchlorate solution as described by Wilcockson [25]. In our hands, DNA prepared in this way was essentially free from glycogen (anthrone method), RNA (orcinol method) and protein of which contamination was always less than 5% (μg protein/μg DNA, Lowry method). We have noticed in our liver preparations from unstarved animals that high concentrations of glycogen (100 μg/ml DNA solution) can occur in our final DNA preparations, if alpha amylase treatment was omitted. We have also observed that such high levels of glycogen contamination of DNA interferes with the poly(U) hybridization on filters (data not shown).

Poly(U) annealing method

We have described elsewhere [7] the essential details of our poly(U) hybridization method and the factors which limit its reproducibility. However, we have now introduced modifications which have reduced much of the variability encountered in our earlier experiments. Approximately 15 μg samples of DNA dissolved in 2 × SSC were denatured by heating at 100°C for 10 min followed by another 10 min with an equal volume of 4 M NaCl. Treating denatured DNA with hot 2 M NaCl insured retention of (dA) rich sequences

and improved on selective removal of any residual contaminating protein [7]. The solutions were cooled immediately in an ice bath, loaded onto nitrocellulose filters (Millipore®) with suction and baked in an oven for 2–4 hr at 80°C. In order to improve on our yield of DNA from smaller tumors, the alcohol precipitated DNA was often collected by centrifugation. These DNA preparations tended to contain lower molecular weight DNA fragments that are normally left behind when the DNA is spooled and thus these DNA solutions retained less on the filters. Previously severely sheared DNA affected the annealing of poly(U) [7]. However, we have compared only DNAs which retained between 75–100% on the filters, and with this level of retention the estimation of (dA) regions was unaffected by the amount of DNA immobilized (data not shown). Commercial [^{3}H]poly(U) (Miles Laboratory, 75 μCi/μM monophosphate) was dissolved in 2 × SSC and adjusted to a final concentration of 6.0 μg/ml with unlabelled poly(U) (Sigma). The DNA containing filters were placed in the [^{3}H]poly(U) solution and annealed for 17 hr at 25°C with continuous mixing from the aid of a magnetic stirrer. Unannealed poly(U) was removed from the filters by ribonuclease A treatment in 2 × SSC at 5 μg/ml for 6 hr at 25°C. It was important, especially in cases where many filters were treated batchwise, to have efficient stirring for both the annealing and RNAase steps if unnecessary variability was to be avoided. The details involving radioactive counting on filters, quantitative determination of DNA and estimation of (dA) "pure" sequences from the % poly(U) annealing to DNA after 6 hr RNAase treatment were unchanged from our earlier studies [7]. Routine poly(U) analyses were replicated 10–30 times. The amount of DNA hybridizing to poly(U) was always determined on the nitrocellulose filters after RNAase treatment and scintillation counting.

T_M fractionation of DNA on hydroxylapatite

The T_M fractionation was carried out as we have described earlier for human DNA [17] except for a few minor details. Native DNA was loaded onto hydroxylapatite in 0.01 M phosphate buffer at 60°C and the A+T rich DNA fraction eluted between 60 and 91°C in 0.12 M phosphate buffer.

Base composition analysis

The method used for base analysis was the same as reported by Wang [26]. Briefly, it is based on the fact that all the bases except adenine react with *N*-bromoacetamide to cause reduction in UV absorption. Standard solutions of dAMP, dCMP, dGMP and dTMP were prepared in 0.5 M H_2SO_4. Since at this acid concentration dAMP and dGMP were depurinated, then the molar absorbancy coefficients used to adjust the final standard concentrations to 0.05 mM were as given: adenine ($E_{270} = 11.56 \times 10^{-3}$), dCMP ($E_{270} = 11.44 \times 10^{-3}$), guanine ($E_{270} = 7.4 \times 10^{-3}$) and dTMP ($E_{270} = 9.58 \times 10^{-3}$). Mixtures of the 4 standard solutions were used to prepare a standard curve ranging from 100% A+T to 100% G+C. It was necessary in our experiments to heat the DNA solutions in 0.5 M H_2SO_4 for 3 hr at 50°C to insure complete depurination and allow accurate estimation of base composition. Other important details such as the preparation and addition of the brominating agent and the calculation of % A+T from the u.v. absorption changes were carried out as described by Wang [26].

RESULTS

A total of 51 rat sarcomas induced by the polycyclic hydrocarbons were pooled into 10 different groups and compared to normal tissues for alterations in the (dA) regions of their DNAs. A quantitative estimation of the (dA) regions in the DNA from normal rat tissue was always the same whether the sources used were embryo, skin or liver + spleen (Table 1). A constant level of (dA) regions in normal somatic cell DNA of the rat has thus provided the analytical basis for its comparison to the (dA) levels in neoplastic DNA. All groups of tumors reported in Table 1 regardless of inducing agent had statistically significant reductions in the (dA) regions of their DNAs. The level of (dA) reductions in the tumor DNAs varied considerably from one tumor group (MC-69) to another (DMBA-17), but there was a tendency for MC- and DMBA-induced tumors to have the greatest (dA) reductions (Table 1). Such heterogeneity in the (dA) alterations of DNA from tumors induced with so structurally similar carcinogens, or even when induced by the same carcinogen (i.e., MC-12 and MC-69), suggests that additional DNA template changes involving in part the (dA) regions, have accompanied those DNA changes responsible for the neoplastic event itself.

One possible way to judge the degree of additional DNA changes which might occur along with those DNA changes necessary for

Table 1. Comparison of normal tissues to polycyclic hydrocarbon-induced tumors in the rat by quantitative analysis of the (dA) regions in their DNAs.

Tumor inducing agent and identity number*	Number of pooled tumors	% poly(U) annealing to DNA†	Average %/tumor/agent‡
Normal rat tissues			
embryo	—	0.147±0.017	—
skin	—	0.145±0.015	—
liver and spleen	—	0.142±0.023	0.145
BP-10	4	0.124±0.005§	—
BP-11	5	0.107±0.005§	—
BP-15	5	0.121±0.019§	0.117
MC-12	6	0.117±0.008§	—
MC-13	7	0.104±0.008§	—
MC-69	4	0.059±0.009§	0.098
DMBA-17	5	0.134±0.008§	—
DMBA-14	6	0.101±0.006§	—
DMBA-18	3	0.112±0.011§	—
DMBA-19	6	0.098±0.010§	0.110

The DNAs from groups of pooled tumors and from normal tissues of the rat were extracted, immobilized onto nitrocellulose filters and annealed to [^{3}H]poly(U) in 2 × SSC at 25°C for 18 hr. Non-specific binding of [^{3}H]-poly(U) was removed by treatment of the filters with 5 μg/ml ribonuclease A dissolved in 2 × SSC for 6 hr (for further details see Ref. 7 and the text).

*BP = 3,4-benzopyrene; MC = 20-methylcholanthrene; DMBA = 7,12-dimethylbenz(a)anthracene.

†For estimation of (dA) regions divide % poly(U) value by 2.

‡%/tumor/agent represents the average % poly(U) annealing to the DNA from all the tumors induced by the same polycyclic hydrocarbon. Hence, the average (dA) alterations expected for a tumor induced by particular polycyclic hydrocarbon in question.

§$P < 0.0005$ (Student's t-test) when compared to the values for normal rat tissues; DMBA-14 ($p < 0.025$); Means ±S.D. are shown.

polycyclic hydrocarbon-induced neoplastic transformation, might be to compare those tumors which contain high frequencies of either similar or different chromosomal aberrations. It has been shown [27, 28] that specific chromosomal abnormalities are associated with tumors induced by the polycyclic hydrocarbons, and some of these abnormalities such as the trisomy A2 can be found in tumors induced by all 3 of the carcinogens BP, MC and DMBA [22, 27]. In Table 2 we have selected BP- and MC-induced tumors which had high incidences of a particular type of chromosomal aberration in the tumor cell populations. This protocol has allowed us to try and standardize the initial amount of DNA damage inflected by the carcinogen at the time of transformation, as evidenced by the similar chromosomal aberrations in the subsequent tumor cell population, and to test whether these chromosomal similarities were specific enough to reflect similar (dA) alterations. Table 2 clearly demonstrates that a high incidence of a distinct chromosomal deviation such as trisomy or the presence of a marker chromosome in the tumor cell population, or even the absence of abnormalities altogether, does not predict the neoplastic-associated (dA) alterations in the DNA. Apparently the presence of other random chromosomal aberrations in the tumor cells must mask any specificity shown by the non-random, distinct chromosomal deviations, at least, as far as any correlation to (dA) alterations are concerned. In fact, support for this interpretation can be drawn from the fact, as we already observed in Table 1, that again there was a rather good correlation to inducing agent; namely, MC-induced tumors all had greater reductions in the (dA) regions of their DNAs than did BP-induced tumors (Table 2). Such a correlation to inducing agent would take into account the total altered genome and not just the chromosomal aberrations which were either specific, non-specific or absent.

We have further pursued our efforts to distinguish the more specific neoplastic-associated (dA) changes from those non-essential DNA changes which might accompany polycyclic hydrocarbon induction of rat sarcomas, by measuring the (dA) levels in A

Table 2. Lack of correlation between the chromosomal aberrations in polycyclic hydrocarbon-induced rat tumors and the (dA) alterations of their DNAs.

Tumor inducing agent and identity number*	Chromosomal aberration specificity	% poly(U) annealing to DNA†
Normal rat tissue	normal	0.145±0.019
BP-1	normal	0.101±0.017‡
BP-2	trisomy A2	0.134±0.017‡
MC-6	trisomy A2	0.055±0.002‡
MC-7	trisomy A2	0.056±0.003‡
BP-3	marker i(2)	0.134±0.011‡
MC-8	marker i(2)	0.070±0.002‡
BP-4	marker t(3;?)	0.118±0.017‡
MC-9	marker t(3;?)	0.054±0.017‡
BP-5	marker t(11;?)	0.111±0.018‡

The DNAs from individual rat tumors which had been karyotyped for any specific chromosomal abnormalities and from normal rat skin and liver were extracted, immobilized onto nitrocellulose filters and annealed to [^{3}H]-poly(U) for estimation of (dA) clusters (for further details see Ref. 7 or text). Additional details of the chromosomal aberration designations are found in Ref. 27.

*BP=3,4-benzopyrene; MC=20methylcholanthrene; The identity numbers in this table correspond to the following identification numbers in Ref. 27 presented in order from top of table to bottom: BP10, BP12, MC8, MC1, BP9, MC2, BP2, MC2+5, BP4.

†For estimation of (dA) regions divide % poly(U) value by 2.

‡$P<0.05$ (Student's t-test) when compared to value for normal rat tissue; Means ±S.D. are shown.

+T rich and G+C rich subfractions of the native tumor DNAs. Table 3 indicates that when the tumor DNAs were eluted from hydroxylapatite in 0.12 M phosphate buffer at 91°C, which was close to the T_M for normal rat DNA (56.5% of the DNA melted), varied amounts of the native tumor DNAs were melted (44.6–73.0%). Although small changes in temperature (<1°C) around the T_M can seriously alter the amount of DNA melting, the results nonetheless suggest base compositional changes in the tumor DNAs. At any rate, the A+T rich fractions of BP, MC and DMBA tumor DNAs all showed significant reductions in their (dA) levels except for MC-12 ($P<0.10$) (Table 3). However, the opposite was true for the G+C rich fractions where only 2 of the 8 tumor groups had

Table 3. Evidence for specificity of (dA) alterations in the DNAs from rat tumors induced by polycyclic hydrocarbons.

Tumor inducing agent and identity number*	Hydroxylapatite thermal elution fractionation of DNA				
	% native DNA melting at 91°C	A+T rich DNA % poly(U) hybrid	A+T rich DNA t-test†	G+C rich DNA % poly(U) hybrid	G+C rich DNA t-test†
Normal rat tissue	56.5	0.136±0.019	—	0.151±0.032	—
BP-10	44.6	0.107±0.019	<0.0005	0.144±0.016	NS
BP-11	62.3	0.121±0.018	<0.025	0.171±0.041	NS
BP-15	67.3	0.095±0.022	<0.0005	0.096±0.017	<0.0005
MC-12	55.0	0.125±0.023	<0.10	0.146±0.028	NS
MC-13	60.5	0.104±0.015	<0.0005	0.140±0.030	NS
MC-69	73.0	0.064±0.017	<0.0005	0.087±0.020	<0.0005
DMBA-14	50.1	0.114±0.020	<0.005	0.158±0.024	NS

The DNAs from groups of pooled tumors and from normal rat liver and embryo were loaded onto hydroxylapatite columns in 0.12 M sodium phosphate buffer at 60°C and the A+T rich DNA fractions collected at 91°C (see text). The A+T and G+C fractions were immobilized onto nitrocellulose filters and annealed to [^{3}H]-poly(U) for estimation of the (dA) regions (for further details see Ref. 7, 18 and text).

*BP=3,4-benzopyrene; MC=20-methylcholanthrene; DMBA=7,12-dimethylbenz(a)anthracene.

†P values for comparison to normal rat tissue; Means ±S.D. are shown; NS=not significant.

significant (dA) alterations (Table 3). These results were interpreted to mean that the majority of (dA) alterations specifically associated to neoplastic transformation induced by the polycyclic hydrocarbons must have occurred primarily in the A+T rich DNA.

The possibility of base compositional changes in the polycyclic hydrocarbon induced tumor DNAs, which was previously suggested from our data in Table 3, was determined for 2, 2 and 4 groups of BP-, MC- and DMBA-induced tumors, respectively. The results in Table 4 indicate rather heterogeneous but statistically significant effects on the base composition of the tumors induced by the polycyclic hydrocarbons, where increases, decreases and no changes at all were observed if comparison was made to normal rat DNA. However, all the groups of tumors examined in Table 4 regardless of whether their base compositions were increased, decreased or unchanged, still had significant reductions in their (dA) levels. As a result, a particular kind of overall base compositional change in native tumor DNA was not a necessary prerequisite for demonstrating the (dA) disturbances associated with polycyclic hydrocarbon-induced carcinogenesis. Another striking parallel to our base analysis data is the fact that the DMBA-induced tumors tended to cause reductions in the % A+T in their native DNAs, whereas BP- and MC-induced tumors tended to cause increases or no changes at all in the % A+T (Table 4).

This study of (dA) alterations in neoplasm was carried out on the rat, but we have previously shown that similar neoplastic-associated (dA) disturbances can be found in the human [17, 18]. Therefore, we have compared the normal human and normal rat genomes to study if their (dA) regions are distributed within their respective genomic DNAs in a similar way. Table 5 demonstrates that (dA) regions can be found in essentially all classes of DNA from the rat and the human. However, the human differs in its (dA) distribution pattern from the rat by having a (dA) enrichment in main band human DNA when compared to the G+C rich and A+T rich human DNA fractions. The rat genome differed by having the same (dA) level in both G+C and main band rat DNA fractions.

DISCUSSION

We have tried to show that rat tumors have small but detectable DNA template changes when compared to normal somatic cell rat DNA. Detection of these base changes have been carried out by a conventional base analysis method [26] and by utilization of a poly(U) probe to measure (dA) rich regions in polypurine rich DNA. In the latter case it

Table 4. The base composition of DNAs from rat tumors induced by polycyclic hydrocarbons and its comparison to (dA) alterations

Tumor inducing agent and identity number*	Base composition analysis n	% A+T	*t*-test	% poly(U) annealing to DNA‡
Normal rat tissue	38	58.6±1.8	—	0.145±0.019
BP-10	12	59.7±2.2	NS	0.124±0.005§
MC-12	10	58.6±1.6	NS	0.117±0.008§
DMBA-14	13	58.4±2.3	NS	0.101±0.006§
BP-11	13	60.0±2.4	<0.05	0.107±0.005§
MC-13	18	60.6±1.6	<0.025	0.104±0.008§
DMBA-17	11	56.7±1.9	<0.008	0.134±0.008§
DMBA-18	15	55.6±2.0	<0.0001	0.098±0.010§
DMBA-19	5	54.9±0.1	<0.0001	0.112±0.011§

The DNAs from groups of pooled tumors and from normal rat liver and embryo were subjected to base composition analysis by measuring the reduction in u.v. absorption following bromination with *N*-bromoacetamide (for further details see Ref. 26 and the text). The same DNAs were also analyzed for (dA) alterations as reported in Table 1.

*BP=3,4-benzopyrene; MC=20-methylcholanthrene; DMBA=7,12-dimethylbenz(a)anthracene.

†*P* values for comparison to normal rat tissues; NS=not significant; Means ±S.D. are shown.

‡For estimation of (dA) regions divide % poly(U) value by 2.

§$P<0.025$ (Student's *t*-test) when compared to normal rat tissue; Means ±S.D. are shown.

Table 5. Genomic distribution of the (dA) regions in rat and human DNA fractional according to base composition in CsCl *gradients.*

Source	% poly(U) annealing to DNA of the pooled CsCl fractions* G+C rich DNA	Main band DNA	A+T rich DNA
Rat	0.169±0.008	0.165±0.011†	0.124±0.015
Human	0.128±0.016	0.162±0.012‡	0.116±0.016

Approximately 500–850 μg of DNA from normal rat and human tissues were mixed with a CsCl solution to an initial density of 1,710 g/cm^3 and centrifuged for 72 hr at 44,000 rev/min in a Ti60 rotor. Twelve drop fractions were collected and then pooled into G+C rich (rat=26.1%, human=31.7%), main band (rat=33.3%, human=29.9%) and A+T rich (rat=40.6%, human=38.4%) fractions of the native DNAs. Each DNA fraction was then annealed to [^{3}H]poly(U) for estimation of the (dA) regions as already described (see Ref. 7 and the text).

*Means ±S.D. are shown.

†Statistically different (*t*-test, $P<0.0005$) from A+T rich DNA but not G+C rich DNA of the rat.

‡Statistically different (*t*-test, $P<0.0005$) from both G+C and A+T rich human DNAs.

can be argued that homopolymeric hybridizations to DNA have inherent methodological disadvantages, especially with formation of poly(U)-DNA hybrids which have low melting temperatures and are thermally more unstable. These disadvantages stem from the fact that the (dA) sequences are quite small in DNA and are close to the minimum stable length for molecular hybrid formation. As a result, we have shown that poly(U)-DNA hybrids are unusually sensitive to protein contamination, glycogen contamination, salt concentration, molecular weight, temperature and ribonuclease digestion [7, unpublished results]. However, we have been aware of these methodological difficulties and have taken them into consideration in our experiments. Nonetheless our results could have been influenced to some degree by such uncontrollable factors as specific tumor-associated nuclease effects on DNA preparation or acidic proteins bound very tightly and specifically to (dA) regions. Indeed these possibilities seem extremely remote explanations. Even if such explanations were proved to be responsible for our experimental observations, then our results would still indicate important biological variations of DNA that are associated to neoplasm.

Our results on the (dA) disturbances in DNAs from polycyclic hydrocarbon-induced rat tumors have indicated that all were significantly altered from normal DNA (dA) levels. There was also quite a heterogeneous distribution in the degree of (dA) changes among the different chemically induced tumor groups and even within the tumor groups induced by the same carcinogen. Nevertheless, there was a trend for the stronger carcinogens (DMBA and MC) to cause greater (dA) changes (Tables 1 and 2). This overall heterogeneity in distribution of (dA) changes among the various tumor groups can probably best be understood when considering the nature of chemically induced neoplasia. In such cases, there is an abnormally high exposure of carcinogen to target cells, and it is quite reasonable to suspect that high levels of DNA damage are inflected. Cells that do transform might carry not only base compositional changes associated with neoplasm but additional base changes not associated with neoplasm, and yet their presence in the genome does not alter cellular metabolism enough to cause a reduced cell survival. These non-neoplastic associated base changes would naturally vary from one tumor to another as a result of varying carcinogen reactivity in the original transforming cells. This type of base compositional change could involve (dA) regions, and if so would explain the observed heterogeneity in neoplastic associated (dA) changes.

Other previous work [20, 21] has established that DMBA is the strongest carcinogen as it induces the highest frequency of tumors or cell transformations at the lowest doses. It has also been shown that DMBA binds more to DNA in cultured cells [29] and in epithelial homogenates [30] than does BP or MC. This is supportive evidence for the increased carcinogenic potential of DMBA when viewed from the mutational theory of cancer. Our data have also been able to distinguish the DNAs from DMBA-induced tumors from those of either BP- or MC-induced tumors. Although BP, MC and

DMBA tumor DNAs all had significant reductions in the (dA) regions of their A+T rich DNA fractions when compared to similar normal DNA fractions (Table 3), only DMBA tumor DNAs were altered in base composition toward a more G+C rich DNA (an indication of preferential DMBA attack in A+T rich DNA, Table 4). Whether any correlation exists between the preference of (dA) alterations to appear in the A+T rich DNA fractions of tumors induced by the polycyclic hydrocarbons (Table 3) and the overall increased carcinogenicity of DMBA, still remains only speculative. However, if such a correlation were proved to be true it would be of importance to the mutational theory of cancer.

We would like to point out that extreme caution should be exercised when extrapolating our results to neoplasms in animal systems other than the rat. Guttman *et al.* [31] have shown that the DNAs from 15 different mammals, analyzed by CsCl-netropsin density gradient centrifugation, contain quite different profiles with respect to their distribution patterns of (dA.dT) clusters. Indeed this has been our experience so far with normal and neoplastic DNAs from the rat and human. If we compared our T_M-DNA fractionation experiments with human neoplastic DNAs [17] to our similar experiments with rat tumor DNAs, then the (dA) regions in the A+T rich fractions of human neoplastic DNAs were increased over those in normal A+T rich human DNA, whereas in the rat tumor A+T rich DNAs the opposite was true (Table 3). Of course there are several reasonable explanations for this discrepancy such as the probable differences in oncogen between the human breast tumors and the rat tumors, or the fact that in the human experiments the T_M fractionation at 88°C yielded a 46% fraction of A+T rich DNA and in the rat experiments the T_M fractionation was carried out at 91°C and yielded a 56.5% fraction of A+T rich DNA. However, still the most likely explanation for the varying neoplastic-associated disturbances observed between the rat and human, is probably correlated to differences in genomic distribution of (dA) regions. The data in Table 5 supports this argument. The (dA) regions in normal rat DNA are distributed equally into the G+C rich and main band DNAs but in the normal human genome they are not.

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Effect of Antileukaemic Chemotherapy on Helper and Suppressor Activity of T Cells on Immunoglobulin Production by B Cells

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Abstract—*Fifteen patients with AML in complete remission, receiving immunotherapy, have been studied for T cell function before and after intermittent maintenance chemotherapy. The regulatory influence of patients' T cells on normal B cells* in vitro *was estimated by IgG production. Immediately after chemotherapy there was a slight reduction of helper activity of patients' T cells detectable when equal numbers of B and T cells were cultured together. This rapidly recovered producing a rebound above that prior to chemotherapy. The suppressive influence of high ratios of purified T cells was not immediately affected by chemotherapy. However, at* 14 *and* 21 days *the suppressive effect of excess T cells appeared to be increased. Until antigen markers of helper and suppressor T cells become available in man it will not be possible to say whether this phenomenon is due to a delayed effect of chemotherapy on suppressor cells or an indirect effect of the changes in helper activity.*

INTRODUCTION

THE REGULATORY influence of T cells on B cell function both as stimulators of antibody production [1] and as depressors of already functioning B cells [2] is well established in mice. There is considerable interest in the occurrence of helper and suppressor T cells in malignancy as these may be important in influencing antibody responses. Antibody responses to tumors might be directly helpful or alternatively harmful because of the production of immune complexes [3].

Ideally it would be desirable to measure helper and suppressor cells which influence specific antibody production to tumor antigens. However, at present in man it is only possible to investigate the ability of human T cells to help and suppress antibody production by normal B cells [4]. The present paper studies the ability of T cells from patients with acute myeloid leukaemia (AML) in remission to influence immunoglobulin production by normal B cells in the presence of pokeweed mitogen. It shows that T cells from these patients have the same ability to help and suppress immunoglubulin production as normal T cells. Intermittent maintenance chemotherapy slightly reduces T cell ability to help but this activity rapidly recovers and a rebound increase above pre-treatment levels occurs.

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MATERIALS AND METHODS

Patients

Fifteen patients with AML, aged 47–70 yr, were studied. They had received standard induction chemotherapy with daunorubicin and cytosine arabinoside [5] at least 5 months previously and most of them were on maintenance chemotherapy consisting of 5 day courses of daunorubicin and cytosine arabinoside alternating with thioguanine and cytosine arabinoside at approximately monthly intervals [6]. Five patients in early relapse with normal peripheral blood received a new combination therapy consisting of vincristine, adriamycin, cytocine arabinoside and prednisolone [7]. None of the patients had abnormal cells present in their peripheral blood at the time of study. Three of these 5 patients had

not received chemotherapy for several months. All the patients except one were on weekly immunotherapy (BCG or BCG plus allogeneic cells) [8], which had been started at least 6 months previously. Heparinized blood samples were obtained immediately before a course of maintenance treatment and between 21 and 28 days after the course. Blood samples were taken sequentially from 4 patients at 7, 14, 21 and 28 days after treatment. Ten normal donors aged 35–50 yr served as controls.

Assay system

Peripheral blood lymphocytes were separated on a Ficoll–Triosil gradient. For rosetting T lymphocytes, sheep red blood cells (2%) were treated with neuraminidase (Behringewerke) 15 μm/ml at 37°C for 25 min and were mixed with lymphocytes (10×10^6) and absorbed foetal calf serum in a ratio 2:1:1 volumes and were centrifuged at 150 ***g*** for 8 min. After 15 min the pelletted cells were gently resuspended, centrifuged again and subsequently incubated for 60 min at room temperature. The E-rosetted lymphocytes were then separated on a second Ficoll-Triosil gradient after centrifugation at 400 ***g*** for 35 min. After centrifugation the Ficoll-Triosil was discarded and the pellet containing E-rosette forming cells (T-cells) and sheep red blood cells was treated with 0.91% ammonium chloride at 37°C for 10 min. The T lymphocytes remaining after lysis of the sheep red blood cells were then washed three times in medium (L15). The composition of T lymphocytes was not checked in all experiments; in 8 cases T cells contained from 89–96% E rosetting cells and less than 1% cells staining with anti-macrophage serum, see Janossy *et al.* [4] and Lee and Oliver [9]. No consistent difference in purity was observed in T cells separated from pre- or post-treatment samples. B lymphocytes were obtained from tonsils of unselected tonsillectomies and were prepared as described by Janossy *et al.* [10]. Purified tonsil B lymphocytes were suspended in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) with 10% foetal calf serum, glutamine, and antibiotics and were set up in cultures in flat-bottomed microplates (Microtest II, tissue culture grade, Flow Laboratories). Varying proportions of the T cells under study were added to B cells maintaining the total number of cells (1×10^6/ml) and the volume (0.25 ml) constant. All cultures were stimulated with 2 μl Pokeweed mitogen (Gibco, lot no. 657412) and incubated at 37°C in the presence of 5% CO_2 for 7 days. IgG was measured in the supernatant by double antibody inhibition radioimmunoassay [11]. The results were expressed in μg of IgG/1×10^6 B cells present at the beginning of culture.

Statistical analysis

Statistical analysis was performed by Student's *t*-test.

RESULTS

When normal purified blood T cells are cultured with normal tonsil B cells at a wide range of ratios, in a standard volume, IgG production by B cells is optimal at a ratio of B:T cells $\simeq$50:50. If the ratio of B:T cells is decreased, a reduction of IgG production per B cell present occurs. Thus typical results were: 90B:10T – 2.6μg/1×10^6 B cells; 80B:20T – 4.4 μg/1×10^6 B cells; 50B:50T – 18.2 μg/1×10^6 B cells; 20B:80T – 4.4 μg/1×10^6 B cells; 10B:90T – 0.4 μg/1×10^6 B cells. On the basis of these results, which are similar to those described by De la Concha *et al.* [12], two types of cultures were used to study T cells from patients and normal controls. Cultures with optimal B:T ratio (50:50) and cultures with excess T cells (B:T ratio 20:80). For ease of presentation the results in normal subjects and the leukaemic patients will be considered separately, although the cultures and assays were carried out in parallel. Table 1 shows that cultures with normal T cells (controls) produced an average 14.0 μg IgG/1×10^6 B cells at the optimal ratio of 50:50. When a ratio of 20B:80T was used IgG production fell to 6.1 μg/1×10^6 B cells which represents a reduction of 58%.

T cells were obtained from patients before and between 21–28 days after the commencement of intermittent maintenance therapy. The results in Table 2 show that T cells before therapy behaved very similarly to normal T cells, at a ratio of 50B:50T. However, at a ratio of 20B:80T there was slightly greater immunoglobulin production in the presence of T cells from patients than from normals. The reduction in IgG production due to excess T cells from patients was only 38%. Three to four weeks after the beginning of therapy the immunoglobulin production at the optimal ratio increased by about 60% and was significantly greater than in normal subjects and the patients before treatment ($P < 0.01$ and < 0.05 respectively). In cultures with excess T cells (20B:80T) IgG production was only slightly increased and the reduction

Table 1. *IgG production* ($\mu g/l \times 10^6$ *B cells) by normal B-cells in culture with normal T-cells*

Controls	B-cells: T-cells 50:50 ($0.125 \times 10^6 + 0.125 \times 10^6$ in 0.25 ml)	B-cells: T-cells 20:80 ($0.05 \times 10^6 + 0.200 \times 10^6$ in 0.25 ml)
1	12.7	2.8
2	17.0	4.8
3	15.4	5.9
4	12.5	4.6
5	10.6	6.0
6	12.2	8.0
7	11.7	4.4
8	15.0	8.2
9	13.0	7.1
10	13.9	9.5
Mean values:	14.3	6.1
Reduction:*	—	58%

$$\text{Reduction*: } 100 \times \frac{\text{IgG production in cultures with 50\% T cells} - \text{IgG production in cultures with 80\%T cells}}{\text{IgG production in cultures with 50\%T cells}}$$

IgG production when purified B and T cells were cultured alone was 1.9 $\mu g/l \times 10^6$ cultured B cells and 0.4 μg respectively (mean values).

due to the presence of excess T cells was 54%, similar to that seen with an excess of normal T cells (58%).

Four patients were studied in more detail to determine the time course of these changes. Figure 1 shows that there was a slight depression of the ability of the T cells to help immunoglobulin production 2 days after completing the course of therapy (day 7) but by day 14 a rebound had begun which peaked by day 21. Using excess T cells (20B:80T) the initial effect of chemotherapy was to enhance the relative and absolute degree of suppression. This peaked on day 14 when there was a 71% reduction in IgG production at a time when helper function was already apparently higher than pretreatment. By contrast on day 28 the reduction due to excess T cells was only 21%.

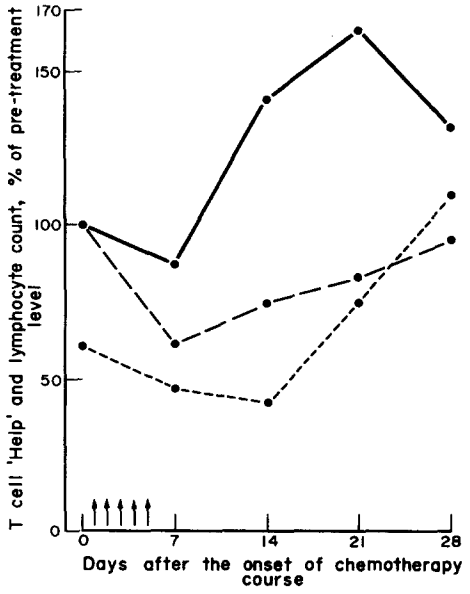

Fig. 1. *IgG production by tonsil B cells cultured with T cells obtained from* 4 *patients, before and* 7, 14, 21 *and* 28 days *after chemotherapy. Cultures were set up with* 50*B:* 50*T* ●—●—● *or* 20*B:* 80*T* ●------●. *The quantity of IgG produced* ($\mu g/l \times 10^6$ *B cells cultured*) *is expressed as a percentage of that produced in cultures containing pretreatment T cells at a ratio of* 50*B:* 50*T. The changes in peripheral blood lymphocyte count over the same time are shown* ●———●. *The peripheral blood lymphocyte counts are mean values for* 15 *patients, the pretreatment value was* 1,815 ± 761 S.D. *lymphocytes/*μl.

IgM production was also measured in 6 experiments. The results showed changes that were very similar to the results for IgG production (data not shown).

DISCUSSION

It is well established that continuous chemotherapy has a more pronounced effect on immune responses than intermittent chemotherapy [13, 14]. However, with intermittent chemotherapy there is evidence for transient loss of T cell function and a more pronounced suppression of B cell function [13]. The previous studies of T cell function have mainly used lymphocyte mitogenic assays and delayed hypersensitivity skin testing. It was the purpose of this study to see whether the intermittent chemotherapy used in the treatment of these patients, which causes about a 30% reduction in total lymphocyte count, effects T cell interaction with B cells using the assay described by Janossy *et al.* [4].

Patients' T cells were cultured with normal

Table 2. IgG production ($\mu g/1 \times 10^6$ B cells) by normal B cells in culture with patients T cells

Patients	Pre-Treatment		After Treatment§	
	B-cells: T-cells 50: 50 ($0.125 \times 10^6 + 0.125 \times 10^6$ in 0.25 ml)	B-cells: T-cells 20: 80 ($0.05 \times 10^6 + 0.20 \times 10^6$ in 0.25 ml)	B-cells: T-cells 50: 50 ($0.125 \times 10^6 + 0.125 \times 10^6$ in 0.25 ml)	B-cells: T-cells 20: 80 ($0.05 \times 10^6 + 0.20 \times 10^6$ in 0.25 ml)
1*	11.5	7.5	32.6	12.3
2*	22.1	9.9	15.0	3.4
3*	14.8	7.3	17.9	3.5
4*	9.0	6.3	29.7	12.3
5†	17.0	4.0	34.6	17.4
6†	10.9	3.1	26.6	21.9
7†	14.0	11.1	35.6	17.9
8†	21.6	15.8	28.9	6.6
9†	9.4	4.2	16.7	9.3
10†	18.9	13.8	24.2	13.5
11‡	23.3	19.9	24.4	9.5
12‡	17.7	16.2	18.5	4.5
13‡	18.3	10.9	34.9	17.5
14‡	21.0	11.4	28.0	14.2
15‡	9.7	6.5	27.1	14.8
Mean values	15.9	9.8	26.3	11.9
Reduction‖	—	38%	—	54%

Treatment:
*Daunorubicin-cytosine arabinoside.
†6-thioguanine-cytosine arabinoside.
‡Adriamycin-vincristine-cytosine arabinoside-prendisolone.
§Blood samples were taken between 21 and 28 days after the commencement of chemotherapy.
‖Reduction

$$100 \times \frac{\text{IgG production in culture with 50\% T cells—IgG production in cultures with 80\% T cells}}{\text{IgG production in cultures with 50\% T cells}}$$

tonsil B cells and the effect on IgG production was measured. Using two ratios of B and T cells it is possible to detect two types of activity. The first one at ratios of 50B:50T is a helper effect of T cells on IgG producing B cells. The second activity seen at ratios of 20B:80T is thought possibly to be a measure of suppressor activity of T cells on IgG producing B cells [12]. It seems unlikely that the suppressive effect of excess T cells is due to macrophages because the number of contaminating macrophages is generally less than 1% [4]. Furthermore in separate experiments an excess of T cells which had been depleted of macrophages by nylon wool filtration or with silica have shown very similar suppression of B cell Ig production (T. A. E. Platts-Mills and M. J. Snajdr, unpublished observations). This regulatory influence of T cells on B cells both as stimulators and as depressors of already functioning B cells is well established in mice [2] and it has been suggested that there are different differentiation antigens on the surface of the T cells involved in the two roles, i.e. LY 1 positive T cells are helpers, LY 2,3 positive T cells are suppressors [15].

The results presented in this paper show that although chemotherapy does cause some suppression of helper activity during the first seven days, there is a rapid recovery with rebound above control levels by 14 days. This is the same as has been seen in studies of other T cell functions in patients on intermittent chemotherapy [13, 14]. In contrast IgG production in the presence of excess T cells (20B:80T) does not increase until 21 days after starting chemotherapy. So that the suppressor activity of T cells appears to be slightly increased following chemotherapy and only begins to decrease on day 21. These data could be interpreted as showing differential susceptibility of two distinct cell types to the effect of chemotherapy. On the other hand when helper activity is maximum at days 14–21 the reduction due to excess T cells is also at a maximum. A clear distinction between these two possibilities will only be established when markers for human T cell subsets become available.

Although these studies were undertaken in acute leukaemia, the findings may apply more generally to other patients receiving intermittent chemotherapy. It seems unlikely that the results are due to immunotherapy as this was started 6 months to 1 yr before the present observations were made and one patient who had received no immunotherapy showed the same rebound phenomenon. It is possible that immunotherapy might have altered the rapidity and the height of the rebound but insufficient cases who had not received immunotherapy have been studied to establish this point. It is clear that intermittent maintenance chemotherapy can cause changes in the balance between helper and suppressor effects of T cells *in vitro*. However, it is also clear that cytotoxic agents in the doses used here have no dramatic effect on the ability of T cells to help *in vitro* IgG production.

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Absence of Cross-Resistance to Alkylating Agents in Cyclophosphamide-Resistant L1210 Leukemia*†

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Abstract—*A cyclophosphamide resistant strain of* L1210 *leukemia was sensitive to treatment with any of eight other alkylating agents containing either β chloroethylamine or aziridine functional moieties including mechlorethamine (nitrogen mustard); Tris(aziridinyl)phosphine sulfide (ThioTEPA); alanine,* 3-[p-[*bis*(2-*chloroethyl*) *amino*] *phenyl*]-*monohydrochloride* (*melphalan*); *four Tris*(*aziridinyl*)*phosphine oxide* (*TEPA*) *analogs and* 1,3,4,5-*Tetra* o-*acetyl*-2-(*di*-2-*chloroethyl*)*amino*-2-*deoxy*-D-*glucopyranose. Cross resistance was virtually absent excepr in the case of o-*[*bis*(1-*aziridinyl*)*phosphinyl*]-*N-hydroxyurethane and melphalan where partial cross resistance was documented.*

INTRODUCTION

THE MOTIVATION leading to this investigation related to our interest in contrasting the properties of 1,3,4,6-tetra-*o*-acetyl-2-(di-2-chloroethyl)amino-2-deoxy-D-glucopyranose (TGM) with other alkylating agents. TGM, like streptozotocin, contains a glucosamine moiety and we previously showed that the schedule dependency of optimal treatment for TGM resembled that of streptozotocin in that the drug is best given on a daily or multiple daily treatment schedule [1]. Human tumors resistant to other nitrosoureas apparently are sensitive to treatment with streptozotocin [2] and this raised the question whether neoplasms resistant to standard alkylating agents could be successfully treated with TGM. In the present investigation we studied cross resistance in a cyclophosphamide (CTX) resistant strain of L1210 leukemia to a number of alkylating agents. Formulae of all the newer drugs used are shown in Fig. 1.

MATERIALS AND METHODS

TGM,*o*-[bis(1-aziridinyl)phosphinyl]-*N*-hydroxy-urethane (AB180), *N*-[bis(1-aziridinyl)phosphinyl]-*o*-acetyl-*N*-hydroxyurethane (AB181) and *o*-[bis(2,2)dimethyl-l-aziridinyl) phosphinyl]-*N*-hydroxyurethane (AB182) were synthesized by Hsiao and Bardos by previously published methods [1, 3]. The preparations of ethyl bis(2,2-dimethyl-l-aziridinyl)phosphinate (AB163) and alanine, 3-[*p*-[bis-(2-chloroethyl)amino]phenyl]-monohydrochloride (PAM) used were those in use for clinical investigation. AB163 was synthesized by Starks Associates, Buffalo, New York. A parenteral preparation of PAM (Burroughs Wellcome, Research Triangle, N.C.) was used. Standard commercial preparations of parenteral forms of all other drugs were used: Mechlorethamine (HN2) (Merck, Sharp & Dohme, West Point, Pa.); Tris(aziridinyl)phosphine sulfide (ThioTEPA) (Lederle Laboratories, Pearl River, N.Y.); CTX (Mead Johnson Laboratories, Evansville, Indiana).

The CTX resistant strain of L1210 leukemia was obtained through the Chemotherapy Department of Southern Research Institute, Birmingham, Alabama, which was

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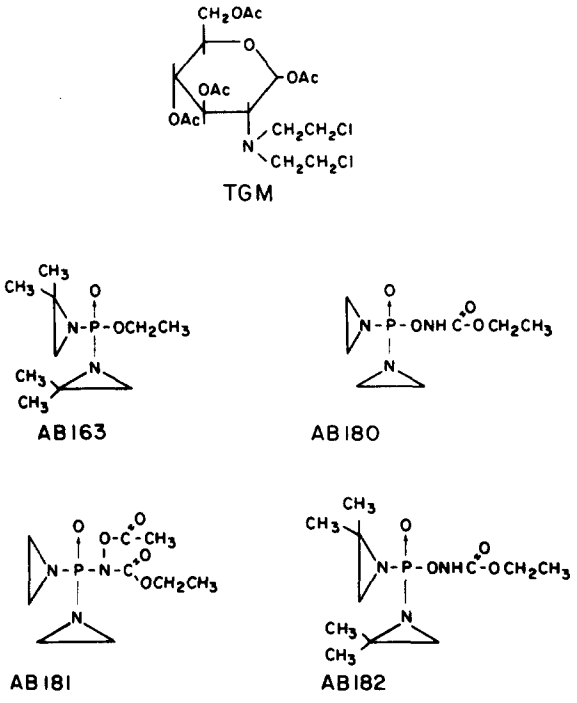

Fig. 1. *Chemical structures of TGM, AB*163, *AB* 180, *AB*181 *and AB*182.

originally developed by William DeWys, University of Rochester, Rochester, New York. His method of inducing tumor resistance and some characteristics of this line have been published [4]. The standard L1210 leukemia utilized was originally obtained from Microbiological Associates, Rockville, Maryland.

Both tumors were maintained in our laboratory by weekly i.p. passage of 10^5 cells into DBA/2 mice. Approximately once each month, CTX was administered to animals bearing the CTX resistant tumor strain to maintain drug resistance. C57BL/6 × DBA/2 (hereafter called B6D2F$_1$) female mice, eight per group, were used for the test animals. Each test animal received 10^5 i.p. tumor cells that led to death of untreated animals in 7–11 days.* For all experiments, groups of mice were kept 8/cage in an air conditioned, light cycle controlled environment. Animals were observed daily for deaths; life span was recorded.

In all experiments, drugs (except TGM) were administered as a single i.p. dose 24 hr after tumor inoculation. TGM was given on a twice daily i.p. schedule for 8 days starting 24 hr after tumor inoculation. These schedules are the known or presumed (based on available evidence) optimum treatment schedules for all drugs utilized. In most cases, duplicate or triplicate trials of each drug in each of the tumor lines were conducted. For statistical analysis, all the data from optimum treatment groups were pooled combining the results from ten experiments which included 7 runs with standard L1210 leukemia and three runs using CTX resistant L1210 leukemia. In order to combine experiments for comparative analysis, only those experiments which utilized a comparable tumor burden were included, e.g. when 10^5 tumor cells injected i.p. were given and when the median life span of the controls fell between 8 and 10 days. Formulation of medication, time of administration and route of administration were identical in the treatment of mice bearing the two tumor lines. The log interval between doses was identical with few exceptions (listed in footnotes in Table 1). This implies that the probability of missing the optimum dose was no greater for one tumor line than for the other.

For statistical analysis, the Kruskal–Wallis one-way analysis of variance by ranks method was used to determine the difference between L1210 and CTX resistant L1210 treatment results. The median test was used to determine differences in results of treatment between pairs of drugs [5]. *P*-values of 0.05 or less were considered significant.

RESULTS

Table 1 lists the optimum results seen for each experiment. The optimum result is based on a calculation of the median life span. (In one instance, the optimal dose would have been different if the number of survivors had been the determining factor.) The raw data from the groups listed in Table 1 have been analyzed as indicated above and the *P*-values obtained in making the various comparisons are listed in Table 2.

Comparison of the standard and CTX resistant L1210 by the method of analysis used indicated that control life span was significantly greater for the CTX resistant tumor. However since animals were observed only daily for deaths the life span difference in these two groups was no greater than the resolution of the time measurement. Consequently, considerable caution should be applied to any conclusions drawn regarding the meaning of this difference.

There was no statistical difference in the

*Four per cent (2 animals) of the standard L1210 leukemia controls lived longer than 11 days.

Table 1. *Comparative activity of alkylating agents against* L1210 *leukemia and cyclophosphamide resistant* L1210 *leukemia*

Tumor*	Drug	Dose range (mg/kg)	Optimal dose† (mg/kg)	40-day survivors/ No. of animals‡	Median life span treated/ control (days)	Increase in life span (%)§
L1210	TGM	—‖	1	1/8	20/8	150
L1210	TGM	—‖	1	0/8	18/8	125
L1210	TGM	0.45–1.01	0.68	2/8	18/8	125
CR L1210	TGM	0.45–1.01	0.68	0/8	17/8.5	100
CR L1210	TGM	0.45–1.01	0.68	2/8	19/10	90
L1210	HN2	2–6.75	3	0/8	14/9	56
L1210	HN2	2.5–3.5¶	2.5	1/8	14/8	75
CR L1210	HN2	1.5–3.38	2.25	0/8	13/8	63
CR L1210	HN2	2.25–5.06	3.38	0/8	15.5/8.5	82
CR L1210	HN2	2.25–5.06	2.25	1/8	14/10	40
L1210	AB181	6–13.5	13.5	4/8	40+/8	400+
CR L1210	AB181	6–13.5	13.5	2/8	18/10	98
CR L1210	AB181	6–13.5	13.5	3/8	19/8.5	98
L1210	PAM	11.3–25.3	16.9	7/8	40+/8	400+
CR L1210	PAM	11.3–25.3	11.3	2/8	19/10	90
CR L1210	PAM	11.3–25.3	16.9	2/8	17.5/8.5	106
CR L1210	PAM	7.5–37.5	11.3	8/8	40+/8	400+
L1210	ThioTEPA	15–33.8	22.5	2/8	18/8	125
CR L1210	ThioTEPA	10–33.8	22.5	0/8	14/8	75
CR L1210	ThioTEPA	15–33.8	22.5	1/8	15/10	50
CR L1210	ThioTEPA	15–33.8	22.5	0/8	14/8.5	65
L1210	AB180	20–45	45	7/8	40+/8	400+
L1210	AB180	24–54	36	6/8	40+/8	400+
L1210	AB180	30–60**	60	4/8	40+/8	400+
CR L1210	AB180	25–56.3	37.5	5/8	40+/10	300+
CR L1210	AB180	37.5–84.4	37.5	3/8	20.5/8.5	141
CR L1210	AB180	25–56.3	37.5	0/8††	18.5/8	131
L1210	AB163	63–135	135	5/8	40+/8	400+
CR L1210	AB163	100–150**	100	5/8	40+/10	300+
L1210	AB182	100–225	225	4/8	40+/8	400+
L1210	AB182	150–300**	300	2/8	18.5/8	131
CR L1210	AB182	100–225	225	0/8	17/8	113
CR L1210	AB182	150–338	338	3/8	18.5/8.5	118
CR L1210	AB182	150–338	225	4/8	40+/10	400+
L1210	CTX	200–450	300	8/8	40+/8	400+
L1210	CTX	135–300	200	7/8	40+/8	400+
CR L1210	CTX	150–338	338	0/8	10/8	25
CR L1210	CTX	225–506	338	0/8	10/8.5	18
CR L1210	CTX	225–506	338	0/8	11/10	10

*L1210 or cyclophosphamide resistant L1210 (CR L1210).

†The optimal dose is based on the highest recorded median life span. When more survivors occurred with a different dose, this is noted. All drugs were given as a single i.p. dose 24 hr after i.p. tumor inoculation of 10^5 cells except TGM was given twice daily for eight days starting 24 hr after tumor inoculation. Injections of TGM were three hours apart. The total cumulative dose of TGM was 16 times the dose given in the Table.

‡Female $B6D2F_1$ tumor bearing mice.

§Percentage increased life span calculated as follows: [Median life span (treated) × 100/Median life span (controls)] −100.

‖Only one treatment group was used in this experiment.

¶The doses used were not at logarithmic intervals. Doses were 2.5, 3 and 3.5 mg/kg.

**Only two treatment groups were used.

††A higher dose (56 mg/kg) produced 2/8 survivors.

Table 2. Statistical analysis of differences in life span in the two lines of L1210 *leukemia**

Treatment (drug)	L1210 median life span (days)	CR L1210 leukaemia† median life span (days)	*P*-value‡
Control	8.0	9.0	0.021§
TGM	18.0	18.0	0.636
HN2	14.0	14.0	0.493
PAM	40.0+	19.5	0.043§
ThioTEPA	18.0	14.0	0.096
AB163	40.0+	40.0+	0.715
AB180	40.0+	20.0	0.041§
AB181	31.0	18.0	0.809
AB182	22.0	17.5	0.229
CTX	40.0+	10.0	<0.001§

*This table combines and summarizes all the data presented in Table 1.
†Cyclophosphamide resistant L1210 leukemia.
‡Using the Kruskal–Wallis one-way analysis of variance by ranks method.
§Significant at $P<0.05$.

life span of animals bearing the two tumor strains when treated with TGM, HN2, AB181, AB182, AB163, or ThioTEPA. Only with treatment using AB180, PAM or CTX was the *P* value less than 0.05. In this tumor which is almost completely resistant to CTX, no cross resistance was detected to any of the other alkylating agents used except AB180 and PAM and in these instances the cross resistance was so incomplete or marginal that 33% of the AB180 treated animals and 42% of the PAM treated animals were cured by a single drug injection given 24 hr after inoculation of 10^5 CTX resistant tumor cells.

To analyze drug potency alterations in the CTX resistant leukemia, the median test [5] was used to evaluate the relative effectiveness of every possible pair of drugs used in treatment of both tumor lines.

Drugs could then be ranked in groups such that drugs in each group were not statistically different in their effect on prolonging the life of leukemic mice but were statistically better or worse than drugs in different groups (see Table 3). It can be seen that in comparing L1210 leukemia with the CTX resistant leukemia, CTX goes from one of the best to the least effective drug while AB180 and PAM are shifted from the most effective to the intermediate grouping. The type of shift seen with AB180 and PAM would be predicted from the knowledge that in this tumor line partial cross resistance to these drugs exists.

DISCUSSION

These results clearly show that in this CTX resistant L1210 tumor there is little cross resistance to the other alkylating agents studied. *P*-values of 0.05 or less for the comparisons in Table 2 denote partial cross resistance (AB180 and PAM), whereas non-significant *P*-values indicate that no cross resistance has been demonstrated.

Our results essentially confirm and extend those of Schabel who studied the activity of PAM but not the other seven alkylating agents in this very same CTX resistant leukemia. He reported it as sensitive to PAM as is L1210 leukemia [6]. He did not, however, utilize a rigid statistical comparison which is responsible for our demonstration of a marginal partial cross resistance.

At the time the studies were done, we were not aware of the published characteristics of this CTX resistant L1210 leukemia. DeWys had shown that these tumor cells repair sublethal damage to the same degree and at the same rate as standard L1210 tumor cells. He suggested either a difference in cellular drug uptake or a difference in intracellular handling of drug as possible

Table 3. Rank order of effectiveness of drugs in L1210 *leukemia and CTX-resistant* L1210 *leukemia**

L1210 leukemia		Cyclophosphamide-resistant L1210 leukemia	
Effectiveness†	Drugs	Effectiveness†	Drugs
		Ineffective	CTX
Low	HN2, ThioTEPA	Low	HN2, ThioTEPA
Intermediate	TGM, AB181, AB182	Intermediate	TGM, AB181, AB182, PAM, AB180
High	PAM, AB163, AB180, CTX	High	AB163

*Using the median test for comparisons as described in the text.
†Effectiveness groupings in the two tumor lines do not necessarily correspond.

mechanisms of resistance [4]. Goldenberg *et al.* studied the mechanism of CTX transport by L5178Y lymphoblasts and identified a two component carrier mediated facilitated diffusion process which was not inhibited by other alkylating agents including HN2, PAM, chlorambucil, isophosphamide or even activated CTX but CTX itself did inhibit the uptake of CTX-^{14}C. They concluded that CTX was transported by an independent mechanism [7]. If loss or damage to such a transport mechanism were the cause of drug resistance one would not expect to see any cross resistance with other alkylating agents. The transport mechanism for HN2 has also been studied and appears to be specific, e.g. not inhibited by other alkylating agents [8] but inhibited by choline, the natural substrate for the carrier [9].

On the contrary, if drug resistant cell lines utilized a mechanism of resistance involving an increased ability to repair DNA damage cross resistance with other alkylating agents, radiation treatment or possibly other types of chemotherapy could result. In at least one instance, a CTX resistant tumor has been shown to be cross resistant to an anti-metabolite, 6-mercaptopurine ribonucleoside [10].

In HN2 resistant L5178Y lymphoblasts, Goldenberg [11] reported that there was a direct correlation between HN2 resistance and percent tetraploidy (and also between HN2 resistance and cellular thiol content).

With polyploidy, there may be increased genetic information to allow for possibly more efficient repair or function in heavily damaged cells.

Mechanisms of intracellular handling of drugs may or may not be specific to the drug. Drug activation mechanisms (as in the case of CTX) are specific to a given drug and its congeners. Drug inactivation mechanisms likely are directed to the functional group and if so cross resistance might be expected.

The stepwise development of resistance in CTX resistant L1210 leukemia suggests that multiple determinants are involved [4] and it seems likely that different resistant cells utilize a different mix of the many possible resistance mechanisms. It would appear that strains of L1210 leukemia can be developed that are cross resistant [12], partially or incompletely cross resistant [13] or virtually non-cross resistant to other alkylating agents.

What is the situation regarding the cross resistance of alkylating agents in man? This can only be answered in a general way. Animal studies have shown a heterogenous situation exists relative to the propensity to the development of resistance to alkylating agents and the evidence on balance at this time would indicate that in L1210 leukemia, which is generally conceded to be the best model for predicting activity in man, cross resistance is likely to be incomplete or almost entirely absent.

The superior activity of the new alkylating agents used in this study, when they are compared with the nearest clinical analog, along with the knowledge that alkylating agents are not alike after all, strongly supports the continued development of the newer drugs in clinical trials.

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Prednisolone Treatment of Hereditary Pig Lymphoma

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Abstract—*Two age-matched pairs of pigs with hereditary lymphoma were treated with prednisolone sodium phosphate in daily intramuscular doses of* 4 mg/kg *on* 5 days/week *for* 5.5 weeks. *One of each pair was then killed and autopsied, the other* 5 weeks *later.*

Treatment resulted in marked reductions in blood mononuclear cell count, serum globulin (chiefly gamma) levels and lymph node, thymus and adrenal size. Increases in PCV (haematocrit), platelet counts and in serum albumin and magnesium levels occurred. The red cell picture continued to improve until two weeks after treatment ceased. All these changes reversed rapidly during the post-treatment period. Neutrophilia occurred only during the fifth week of treatment.

Serum Na, K, Ca, *chloride and inorganic phosphate levels showed no treatment-related changes.*

The skin hypersensitivity reaction to DNFB, absent before treatment, became positive during and after prednisolone treatment but was never as marked as in sensitised normal pigs.

INTRODUCTION

FOLLOWING the establishment of 4 mg/kg as a suitable dose of prednisolone for pigs [1], an experiment was set up to determine the effects of the drug on cases of hereditary lymphoma of pigs [2] and to establish whether these effects were comparable to those occurring in lymphoid neoplasia of man.

MATERIALS AND METHODS

Two pairs of pigs numbered 30, 37 and 52, 56, which were 6 and 5 months old respectively at the start of the experiment, were used. The latter pair were littermates. All were female and affected with lymphoma which had been diagnosed between 8 and 10 weeks of age.

The same monitoring methods were used as in the corresponding experiment on normal pigs [1] except that all four pigs received the same dose of prednisolone sodium phosphate (Codelsol, M.S.D.), namely 4 mg/kg administered intramuscularly once daily 5 days/week for 5.5 weeks. In addition, a bone marrow core biopsy sample was taken from the wing of the ileum before treatment commenced as well as prior to slaughter. The expiratory girth at the level of the umbilicus and the length and breadth of the inguinal and precrural lymph nodes were measured once weekly. The lymph node measurements were taken with ball-pointed calipers without compressing the node and without lifting it into a fold of skin.

Since it is not always easy to differentiate the various types of mononuclear cells in routine smears from lymphoma cases, differential counts were based on "total mononuclear cells".

All pigs were given supportive therapy throughout the experiment with a vitamin-mineral feed supplement (Nutrequin, Berk). During prednisolone treatment, they also received framycetin sulphate (Feed Additive, C. Vet.) at prophylactic dose level.

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At the end of 5.5 weeks treatment, one pig of each age pair was killed (Group I, pigs 30 and 56). Treatment of the survivors (Group II, pigs 37 and 52) was tailed off during the succeeding three days and they were killed 5 weeks later.

Pig 54, an unaffected littermate of the younger age pair, whose only abnormality was a congenital right deviation of the nasal bones and nasal septum, was killed as an untreated control two days after its Group I sibling.

Further autopsy controls were provided by the use of data from 17 untreated lymphoma cases which had died or had been killed at comparable ages to the treated pigs.

RESULTS

Clinical observations

Before the commencement of treatment, all four pigs ate fairly well, but they were less active and easier to handle than normal pigs of similar size. Weight gains were poor. Within a few days of starting treatment, there was a marked improvement in appetite and general fitness and the pigs became much more lively. Weight gains were not improved during treatment (Table 1). All pigs showed polydipsia and polyuria but had normal faeces. Rectal temperatures varied within the normal range (37.5–39.7°C) apart from a period of febrile response (up to 40.1°C) in two pigs associated with infected abrasions and bite wounds.

During prednisolone treatment, the lymph nodes decreased in size. The length of the left inguinal node was taken as representative since both lengths and breadths of the other nodes showed similar changes. Its mean length in the 4 pigs was 66 ± S.D. 5.7 mm before treatment. After 4 weeks of treatment, it was 45.5 ± 4.7 mm, a decrease of 31%. During the rest of the treatment period, the glands were too soft to be accurately measured. Five weeks after treatment was withdrawn, the mean measurement in the two remaining pigs was 64.5 ± 6.7 mm, an increase of 41.8%, these measurements being compara-

Table 1. Body weights (kg) *and relative weights of organs* (cg/kg *body weight*) *of individual lymphomatous pigs treated with prednisolone compared with an untreated normal (non-lymphomatous) littermate of two of them and 17 untreated lymphoma cases of the same age-group.*

	Normal	Lymphoma cases				
	Untreated	Treated				Untreated
		Group I		Group II		
	Pig No. 54	Pig No. 30	Pig No. 56	Pig no. 37	Pig No. 52	Mean (range) of 17 pigs
Age at death (days)	221	237	219	272	244	231 (200 to 280)
Body weight (kg):						
Pretreatment	75.0	34.0	25.0	28.5	22.0	41 (23 to 83)
End of treatment	—	36.0	31.0	29.0	24.5	—
End of experiment	—	—	—	38.0	30.5	—
Lymph nodes:						
Mesenteric and colonic	267	764	565	5368	2836	4177 (646 to 9906)
Gastric and splenic	28	230	336	1687	1115	1960 (740 to 3210)
Bronchial group	7	25	23	77	84	217 (38 to 919)
Total splanchnic	302	1019	924	7132	4035	6261 (1662 to 12670)
Head group	25	61	62	143	237	163 (45 to 286)
Iliac group	7	13	11	38	75	36 (11 to 67)
Prescapular	2	5	4	12	15	15 (4 to 47)
Precrural	3	13	7	27	35	23 (7 to 39)
Inguinal	8	25	20	47	64	51 (18 to 156)
Popliteal	1	3	3	6	26	11 (2 to 16)
Total superficial	46	120	107	273	452	290 (93 to 476)
Thymus	100	31	21	67	42	18 (0 to 67)
Spleen	373	500	242	1250	1197	2156 (1030 to 7800)
Liver	2193	5069	4194	4711	5607	6288 (3120 to 9270)
Kidney	167	325	209	263	262	331 (180 to 443)
Adrenal	3	4	4	7	5	11 (5 to 28)

In the case of bilateral organs only one side was weighed, except kidney and adrenal which are mean values.

ble to those at the beginning of the experiment. The pot-bellied appearance associated with the disease increased during the treatment period, despite the fact that girth measurements made while the animals lay on their backs remained fairly steady in three pigs and actually decreased progressively from 880 to 800 mm in the fourth (pig 37). There was no clinical evidence of ascites; in particular, little or no fluid could be detected by paracentesis abdominis. During the post-treatment period, the two surviving pigs showed increases of 110 mm (pig 37) and 100 mm (pig 52) in girth measurement which were associated with increases in body weight (Table 1).

Haematology

The most spectacular haematological effect was on the mononuclear cells (Fig. 1) whose value dropped from an overall pretreatment mean of $20.5 \pm 5.6 \times 10^9/l$ to a nadir of $4.1 \pm 0.5 \times 10^9/l$ during the fifth week of treatment when no recognisable tumour cells were observed in the peripheral blood. By the end of treatment, the mononuclear cells had risen to $7.4 \pm 2.2 \times 10^9/l$. During the next five weeks, the mononuclear counts of the two surviving pigs rose progressively to approximately pretreatment levels.

During the pretreatment period, absolute neutrophil counts fluctuated widely in individual pigs, with an overall mean of $9.7 \pm 6.7 \times 10^9/l$ (Fig. 1). During the second week of treatment, this dropped to $6.2 \pm 2.7 \times 10^9/l$, rising to $15.6 \pm 2.5 \times 10^9/l$ during the fifth week and falling again to $9.5 \pm 1.4 \times 10^9/l$ at the end of treatment. This fall continued in the two Group II pigs during the post-treatment period, reaching $3.6 \pm 0.4 \times 10^9/l$ 5 weeks after treatment ceased.

Two weeks before treatment started, all four pigs had low thrombocyte counts (mean $99 \pm 20 \times 10^9/l$ (Fig. 1). By the fifth week of treatment, the mean had risen to $359 \pm 19 \times 10^9/l$. During the last week, it fell to $283 \pm 56 \times 10^9/l$. Four weeks later, the 2 surviving pigs had a mean count of $206 \pm 86 \times 10^9/l$.

Eosinophil counts remained at a low level throughout the experiment in all pigs except No. 37 (Group II) in which they fluctuated considerably.

The packed cell volume (Fig. 2) tended to fall during the pretreatment period but rose rapidly from a mean of 0.26 ± 0.01 at the commencement of treatment to 0.30 ± 0.02 three weeks later, remaining at about this level until the second week after treatment ceased. During the last three weeks of the experiment there was a rapid fall to 0.24 ± 0.03. RBC and haemoglobin showed similar changes.

The only discernible treatment-related effects on the derived red cell indices were in Group II where, during the post-treatment period, the mean cell volume rose from 51.0 ± 1.6 fl to 59.1 ± 5.1 fl and the mean cell haemoglobin concentration dropped from 35.2 ± 2.7 to 31.9 ± 1.4 g/dl.

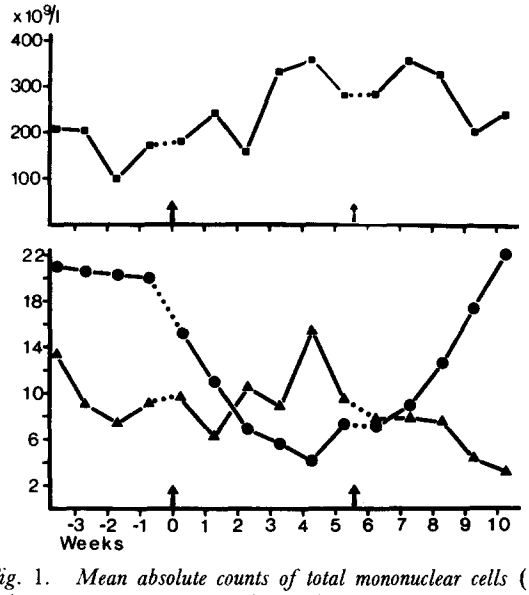

Fig. 1. Mean absolute counts of total mononuclear cells (●–●), neutrophil granulocytes (▲–▲) and platelets (■–■) of lymphomatous pigs before, during and after treatment with prednisolone. Arrows indicate beginning and end of treatment.

Serum biochemistry

The mean serum magnesium concentration, which had remained fairly steady at an overall mean of 0.84 ± 0.05 mmole/l during the pretreatment period, showed upward fluc-

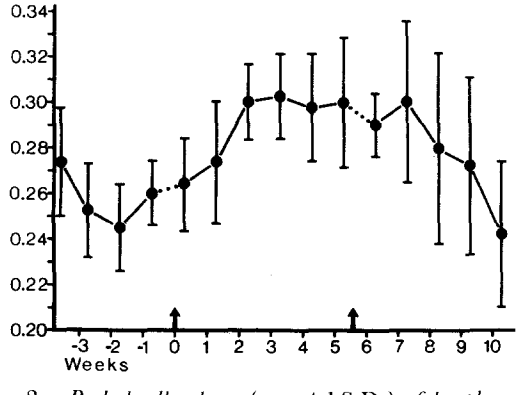

Fig. 2. Packed cell volume (mean ± 1 S.D.) of lymphomatous pigs before, during and after treatment with prednisolone. Arrows indicate beginning and end of treatment.

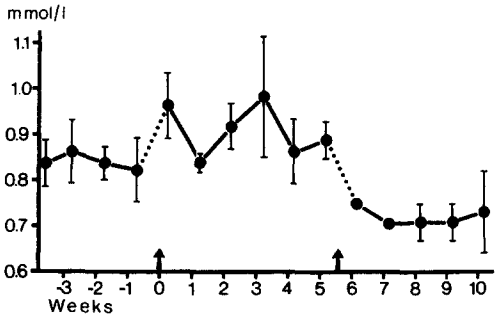

Fig. 3. Serum magnesium (mean ± 1 S.D.) of lymphomatous pigs before, during and after treatment with prednisolone. Arrows represent beginning and end of treatment.

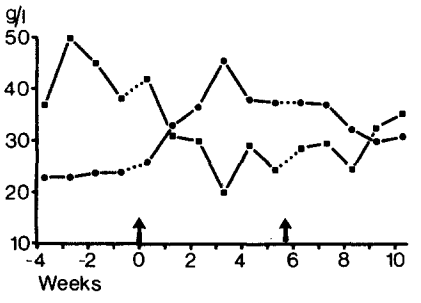

Fig. 4. Serum albumin (●–●) and serum globulin (■–■) of lymphomatous pigs before, during and after treatment with prednisolone. Arrows represent beginning and end of treatment.

tuations during treatment (Fig. 3) but remained within the normal range for the pig (0.8–1.25 mmole/l). After treatment ceased, it fell immediately in the two surviving pigs to 0.75 mmole/l and remained below that level during the rest of the experiment. No signs of hypomagnesaemic tetany were observed.

The mean serum potassium concentration remained unchanged throughout the experiment (Table 2). The mean serum sodium, calcium, chloride and inorganic phosphate concentrations all showed small progressive increases during the course of the experiment (Table 2).

The total serum globulin level (Fig. 4) fluctuated considerably before treatment having an overall mean of 43.1 ± 7.9 g/l. During treatment it fell sharply, reaching a nadir with a mean of 21.0 ± 5.2 g/l during the fourth week. From then on it tended to rise again but within the period of observation, never reached the pretreatment level.

The mean serum albumin level (Fig. 4) remained relatively steady before treatment with an overall value of 23.6 ± 4.0 g/l. During treatment it showed changes more or less complementary to those in globulin. At its peak during the fourth week it had a mean of 46.0 ± 3.9 g/l.

These effects were exaggerated when the albumin/globulin ratio was calculated. Before treatment this had an overall mean of 0.57 ± 0.17. The ratio increased rapidly during treatment reaching a peak during the fourth week with a mean of 2.28 ± 0.5. Thereafter it fell again but remained above unity until the last two weeks of the experiment.

Electrophoretic analysis showed that the drop in globulin during treatment was attributable essentially to a 50% reduction in the gamma fraction (Table 3).

Cell-mediated immunity

Before therapy began, the skin hypersensitivity reaction to 10% DNFB was minimal or absent in all four lymphomatous pigs. During and after therapy, a response of score 2 (erythema and swelling) was apparent (Table 4). The reaction of the untreated non-lymphomatous pig was much more severe (large ulcerations, score 4).

Table 2. Serum inorganic elements (mmole/l) *of lymphoma cases treated with prednisolone*

	A	B	C
Number of pigs	4	4	2
Mg	0.84 ± 0.05	0.91 ± 0.04	0.72 ± 0.01
K	4.84 ± 0.20	4.84 ± 0.46	4.89 ± 0.07
Na	137.25 ± 1.88	142.65 ± 1.05	147.80 ± 4.50
Cl	90.44 ± 1.14	92.37 ± 0.98	94.40 ± 0.60
Ca	2.62 ± 0.10	2.70 ± 0.09	2.74 ± 0.03
PO_4	2.07 ± 0.10	2.02 ± 0.18	2.34 ± 0.23

Mean and S.D. were calculated for all samples taken during A: pre-treatment; B: treatment, and C: post-treatment periods.

Table 3. *Mean* ± S.D. (g/l) *of serum globulin fractions obtained by electrophoresis from lymphoma cases treated with prednisolone.*

	A	B	C	D
Number of pigs	4	2*	4	2
α_1	0.9 ± 0.7	0.45 ± 0.2	0.9 ± 0.4	12.5 ± 1.1
α_2	10.1 ± 2.4	9.10 ± 0.3	9.5 ± 1.1	
β	9.3 ± 2.4	8.75 ± 2.1	10.5 ± 2.4	13.7 ± 3.4
γ	15.7 ± 3.3	7.40 ± 0.6	7.2 ± 1.7	13.4 ± 1.9

A: at commencement of treatment; B; during fourth week of treatment when total globulin value was at its lowest; C: at end of treatment; and D: five weeks later.
*2 samples lost.

Table 4. *Skin hypersensitivity reactions of individual lymphomatous pigs to* 10% *DNFB before, during and after treatment with prednisolone, compared with the reaction of an untreated normal (non-lymphomatous) littermate of two of them*

	Pig No.	At sensitisation	Pretreatment	During last week of treatment	1 month post-treatment
Group I	30	3	0	2	—
	56	1	0	2	—
Group II	37	0	0	2	2
	52	1	1	2	2
Normal	54	1	4	—	—

0, negative; 1, slight erythema; 2, erythema and swelling; 3, small ulcerations; 4, large ulcerations.

Post-mortem and histological findings

Group I pigs resembled normal animals except that the lymph nodes were slightly enlarged. As in untreated lymphoma, the cortex and medulla were not distinguishable on the cut surface but, unlike such cases, the nodes were firm not soft.

Group II pigs showed lesions typical of advanced, untreated cases of the disease as described by Head *et al.* [2], except for the thymus and alimentary tract (q.v. below).

The relative weights of organs are summarised in Table 1. In Group I, treatment had reduced the relative weights of the splanchnic lymph nodes to well below the minimum value of the 17 untreated cases, but they were still about three times greater than those of the untreated normal pig. In Group II, the withdrawal of treatment resulted in marked recrudescence so that the splanchnic nodes were nearly 6 times larger than in Group I and approximated to the mean value of the untreated cases. The increase in relative weight was greatest in the mesenteric and colonic nodes and least in the bronchial group.

Similar, but less spectacular effects were observed in the superficial nodes both individually and as a group.

On histological examination, treatment in Group I had caused a depletion of cells in the paracortical region of the lymph nodes and the few nodules found had reverted to primary small structures (Figs. 5, 6 and 7). The increase in size of the nodes was due to paracortical tissue in which a few probable tumour cells were seen, but none in mitosis. This area in the gastric and mesenteric nodes had much fibrin deposition. Regrowth of tumour in Group II nodes resulted in replacement of normal structure almost completely by tumour cells and some small lymphocytes, except for a few small nodules (Figs. 5 and 8).

The inguinal nodes of Group I were unique in that one pole showed lymphoid depletion described above but the other pole was replaced by tumour so that it resembled a Group II node.

The relative weight of the thymus was about twice as great in Group II as in Group I. All were above the average for the un-

treated cases but none was as high as the untreated normal pig. Histologically, the thymuses of Group I pigs were reduced to lobules of adipose tissue with occasional epithelial cells and groups of up to 50 lymphocytes. The recovery of normal cortical and medullary structure when treatment was stopped resembled that seen in prednisolone treated normal pigs [1], but the more rapidly regrowing tumour had caused some thymus suppression in pig 52.

The effects on the splenic relative weights were similar to those on the lymph nodes, so that histologically the Group I white pulp showed no nodules and the red pulp, though large, was depleted of cells (Fig. 9). Five weeks without treatment allowed tumour to regrow, nodules to reform and extramedullary haemopoiesis to develop in Group II so that the spleen resembled that of a typical late stage untreated lymphosarcoma (Fig. 10).

When bone marrow biopsies were taken immediately prior to slaughter, they were found to be representative of the picture in the decalcified bone sections. The pretreatment biopsies all showed tumour tissue but in the post-treatment samples no tumour cell masses were found, although there were more haemopoietic cells and less adipose tissue than normal. In the Group II post-mortem samples, there seemed to be an increase in normoblast foci, a reduction in megakaryocytes and large islands of tumour cells. Whereas the decalcified lumbar vertebral body and wing of the ilium are normal sites of haemopoiesis, the marrow of the femoral shaft is a fat store in this age of pig. It it noteworthy, therefore, that it was haemopoietic in Group I and both haemopoietic and neoplastic in Group II pigs.

In contrast to other lymphoid sites, the Group I pigs showed large reaction centres in the stomach, Peyer's patches and caecum, particularly in pig 56. Moreover, in the sites occupied by tumour in Group II pigs, there was a sparsely cellular zone with no obvious tumour cells in mitosis.

In some organs, e.g. the ovarian stroma and the adventitia of both the arcuate vessels of the kidney and the blood vessels of the fat of the vertebral canal, a tumour lymphocyte infiltrate was present in Group II but absent in Group I. In other organs of Group I some foci of small lymphocytes were present, whereas they were absent (e.g. periportal) or smaller (e.g. peribronchial) in normal pigs. These areas in Group II animals were occupied by large proliferating tumour masses.

DISCUSSION

There are three main areas of interest in these results: firstly the effect on the marrow in which tumour was reduced but haemopoeisis was stimulated; secondly the effect on serum biochemistry, in particular the changes in albumin/globulin ratio and in magnesium levels; thirdly the differential sensitivity of tumour cells in different organs.

The percentage reduction in circulating mononuclear cells was twice as great as that observed even at the highest dose level of 6 mg/kg in normal pigs [1]. This is in keeping with the observation of Schrek [3] that in man leukaemic lymphocytes were more sensitive to steroids than were normal lymphocytes.

The observed reduction in total serum globulin and increase in serum albumin during treatment was to be anticipated from the observation in man that control of symptoms by corticosteroid therapy is usually associated with such an effect [4]. The reduction in globulin may have resulted from steroid lympholysis primarily of tumour cells which were producing an excess of gamma globulin. However, we have not been able to demonstrate that the high level of gamma globulin found in lymphoma pigs is produced by the tumour cells. Indeed, these appear to be neither T nor B cells since the percentage of cells forming rosettes with sheep RBC and of cells showing fluorescence for immunoglobulin is very low compared with normal pigs (unpublished data). It may be that the gamma globulin came from normal B cells in response to the presence of the tumour. These cells were then involved in the steroid lympholysis or reduced their production of globulin as the tumour disappeared, or both.

The increase in serum albumin levels during treatment appears to result from an osmotic mechanism acting through the hepatic interstitial fluid so that the production of albumin by the liver is inversely related to the globulin concentration [5]. Increased albumin production would be helped by prednisolone-induced improvement in appetite, resulting in increased protein uptake and improved utilisation following reduced interference with organ function by the tumour or its products.

The suspicion that in normal pigs prednisolone produced a nadir in total serum globulin values before the end of treatment [1] was borne out in the lymphoma pigs in which it was accompanied by a peak in the serum albumin. These effects and the nadir in

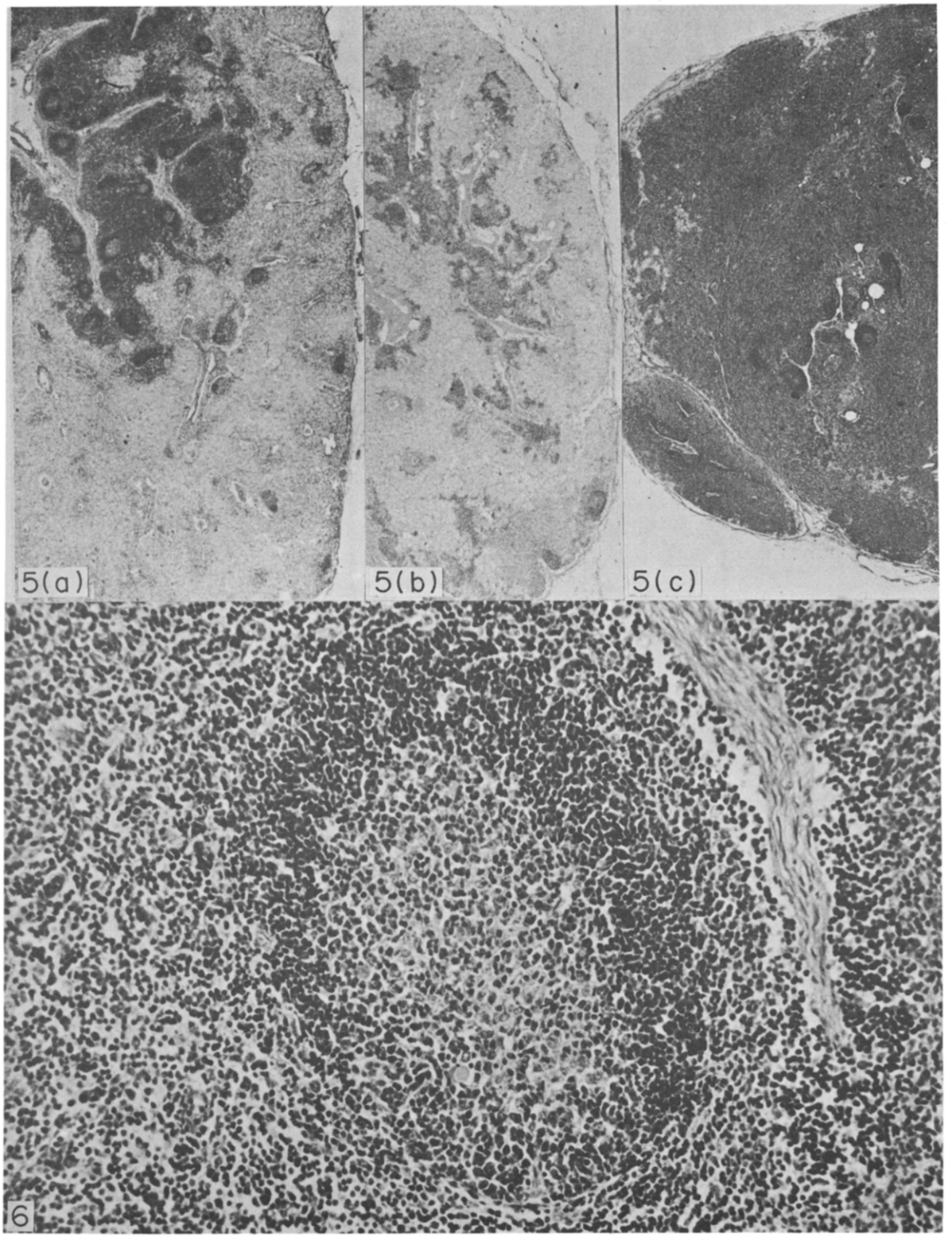

Fig. 5. Prescapular lymph nodes. a, pig 54 normal control; b, lymphoma pig 30 at the end of treatment; c, lymphoma pig 37 at the end of the post-treatment period. Note reduction in the number of cortical nodules and density of paracortical zone in b. In c the medulla is virtually obliterated but some cortical nodules have reappeared. H&E × 10.

Fig. 6. Mesenteric lymph node from pig 54, normal control. Note trabecula on right with surrounding sinus, secondary cortical nodule with reaction centre in the middle and paracortical zone on the left. Compare with Figs. 7 and 8. H&E × 10.

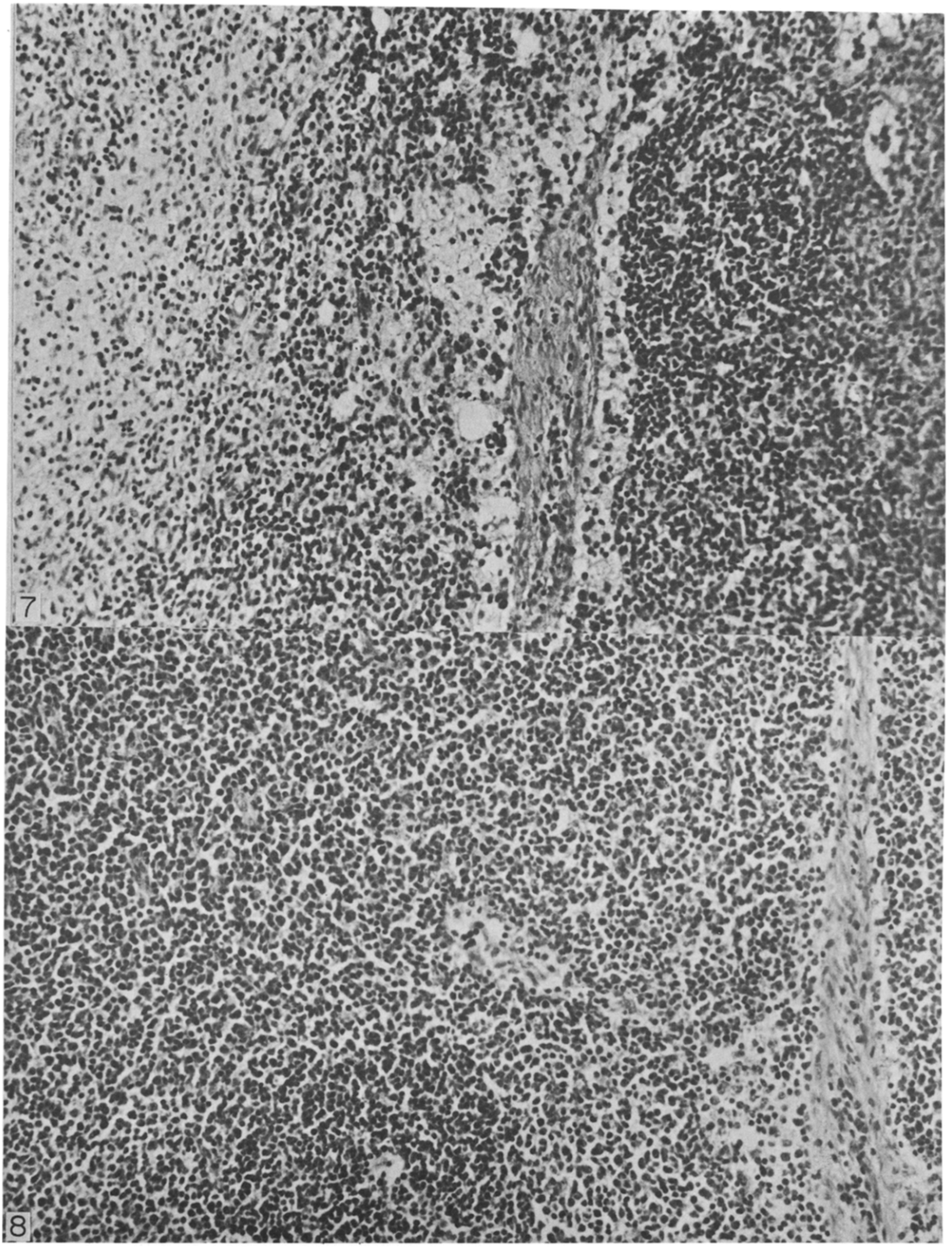

Fig. 7. *Mesenteric lymph node from lymphoma pig* 30 *at the end of treatment. Note trabecula in centre surrounded by dilated sinus, poorly defined primary cortical nodules on right and depleted paracortex on the left. Compare with Figs.* 6 *and* 8. *H&E* × 500.

Fig. 8. *Mesenteric lymph node from lymphoma pig* 52 *at the end of the post-treatment period. Note that tumour recrudescence replaces most of the normal architecture but a trabecula is visible on the right and a poorly defined primary nodule at the bottom centre. Compare with Figs.* 6 *and* 7. *H&E* × 500.

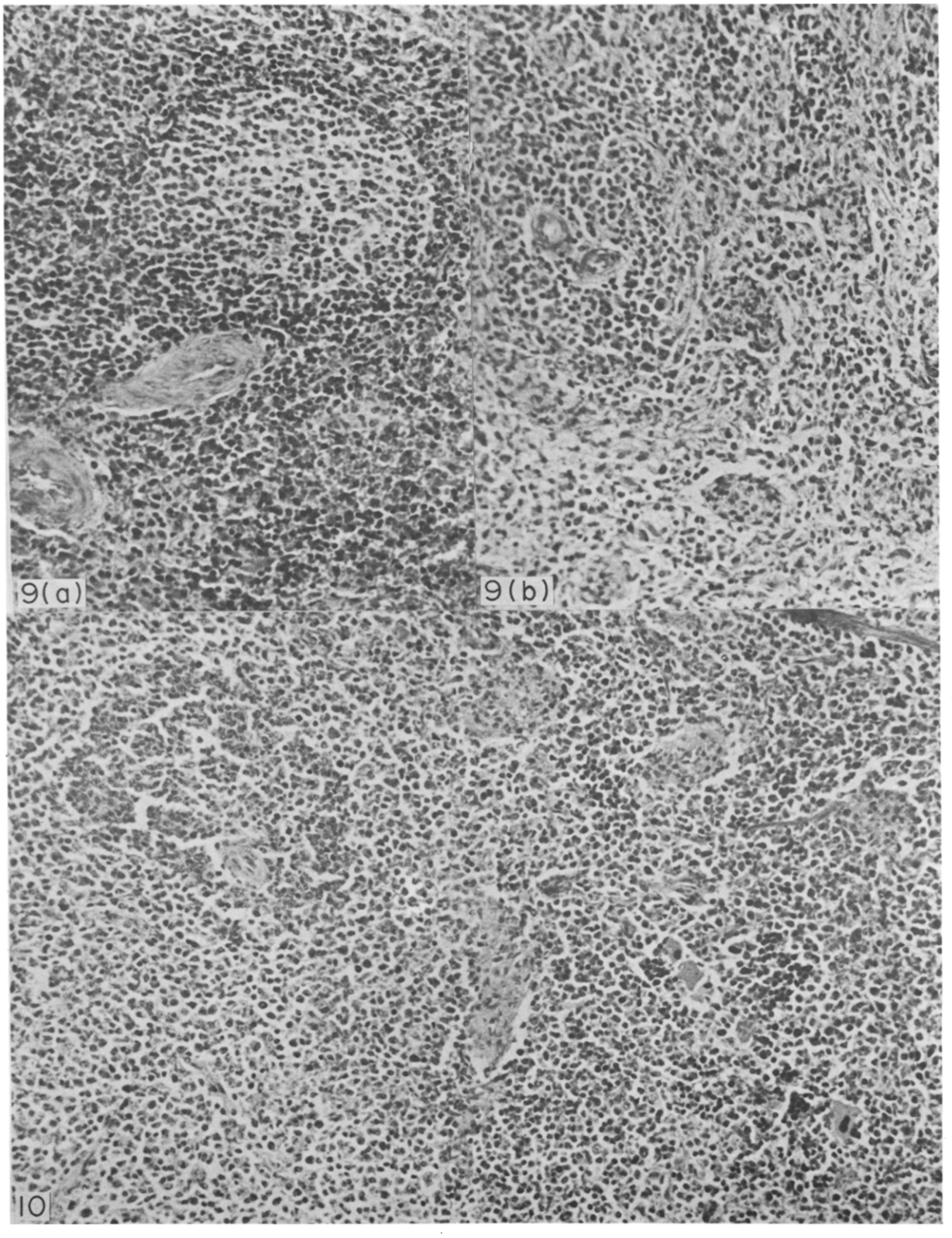

Fig. 9. *Spleens from a, pig* 54, *normal control and b, lymphoma pig* 56 *at the end of treatment. Note the disappearance of secondary nodule and depletion of both red and white pulp in the treated case. Compare with Fig.* 10. *H&E* × 500.

Fig. 10. *Spleen from lymphoma pig* 52 *at the end of the post-treatment period. Note regenerating white pulp top left surrounded by tumour tissue; on the right small dark nuclei of normoblasts and some megakaryocytes can be seen. Compare with Fig.* 9. *H&E* × 500.

mononuclear cells and peaks in neutrophils and platelets during the fifth week of treatment suggest that thereafter the effects of the treatment began to decline. Shaw *et al.* [6] observed that the effects of prednisolone on chronic lymphocytic leukaemia of man were transient and Rosenthal *et al.* [7] reported that remissions obtained in lymphocytic leukaemias of children and other lymphoid neoplasia with ACTH or cortisone were of short duration. This waning of effect may be due to suppression of the hypothalamic–pituitary-adrenal axis [8]. Certainly, the mean relative weight of the adrenals of Group I pigs at the end of treatment was only two-thirds that of Group II 5 weeks after withdrawal and only one-third that of the 17 untreated lymphoma cases (Table 1).

The continued improvement in the red cell picture into the second week after treatment was discontinued might be due to a stimulating effect of prednisolone on early red cell precursors in the marrow so that the resulting increased population of maturing cells in the "pipeline" continued to appear in the peripheral blood for some time.

Increased levels of serum magnesium (up to 1.85 mmole/l which is within the cardiotoxic range) have been observed during treatment of human patients with leukaemia and have been attributed to the release of the element from damaged malignant cells [9]. The reduction to subnormal levels observed in our lymphoma pigs once treatment was withdrawn may be associated with increased uptake of the element from the blood stream by the rapidly regrowing tumour.

Such a mechanism would be expected to affect serum potassium levels similarly [10], but these remained unchanged throughout the experiment. Potassium and calcium loss only result from high dosage or prolonged treatment with corticosteroids [9].

Although the average skin hypersensitivity reaction score of 2 was not as high as that observed in the normal untreated pig, it was higher than that observed in untreated lymphoma pigs (unpublished data). These reactions show that prednisolone had a slightly beneficial effect on the cell-mediated response of the treated lymphoma pigs. This response is unlike that observed in normal pigs receiving prednisolone, where the effect was to suppress the hypersensitivity reaction [1].

The absence of increases in body weight during treatment and the increases observed in Group II after its withdrawal are in keeping with the known growth-depressive effects of prednisolone [11]. It is not clear, however, to what extent these effects are attributable in lymphomatous pigs to changes in tumour mass as reflected in the superficial lymph node measurements and post-mortem findings.

The problem of identifying isolated non-dividing tumour cells sparsely scattered through a population of other lymphoid cells remains unsolved. It is, therefore, difficult to state whether treatment destroyed all the tumour cells in an area or reduced their number and mitotic rate so that they were not detectable by conventional histology. It is clear from our results, however, that tumour growth was suppressed to different degrees in different sites in the body, e.g. completely in the kidney but not in the gastro-intestinal tract. Even in a single organ only localised suppression was sometimes achieved, e.g. the inguinal lymph node.

In the early drug-induced remission period, one might expect to see the supporting connective tissue stroma of the tumour now depleted of tumour cells. The gastric and splenic lymph nodes, which are the organs most enlarged in the untreated case, certainly showed a zone of fibres and blood vessels between the paracortical area and the medulla which would fit this concept. This, and comparable areas in other lymphoid organs, often showed fibrin deposition, suggesting some defect in the integrity of the blood vascular system. In these areas, large "blast" cells were seen which may have been unmasked reticuloendothelial cells or possibly tumour cells prevented from progressing through mitosis.

It is clear from the foregoing discussion that hereditary lymphoma of pigs is a good model for comparison with the human. In addition to changes associated with prednisolone treatment that were predicted from previous work (e.g. red and white cell parameters, albumin/globulin ratio and weight gain) new features have come to light such as magnesium fluctuations, differential tumour sensitivities and correction of anergy. These open up new avenues of research and may lead to changes in our fundamental thinking about lymphoid neoplasia.

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Osteosarcoma of the Long Bones

Clinical Observations and Experiences in the Netherlands

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Abstract—*The diagnostic and clinical aspects of* 176 *patients with osteosarcoma of the extremities, treated during the years* 1962–1969 *are retrospectively reviewed. A classification based on the radiographic appearance of the tumour was made. A specific method for measurement of the primary tumour is defined. In the majority of cases* (75%) *the planned management was by primary irradiation and selective delayed amputation. The* 3- *and* 5-*yr survival rates are* 30 *and* 22% *respectively. Prognosis was influenced by histology and radiographic characteristics. The local palliation obtained by radiotherapy was not adequate to justify delay in amputation. For that reason and in view of the results obtained so far by elective treatment (prophylactic irradiation of the lungs and chemotherapy), immediate ablative surgery is recommended as the treatment of choice.*

INTRODUCTION

In 1953 a study group for bone tumours was formed in the Netherlands, the members of which included radiologists, pathologists, orthopaedic and general surgeons. This committee which later came to be called the Netherlands Bone Tumour Registry (NBTR) initially collected sections of bone tumours and data concerning the patients from the archives of departments of pathology. Files were established as soon as complete case histories were available. After some time requests for advice concerning diagnosis and treatment gradually increased. To day the NBTR has acted for more than 20 yr as a reference centre for bone tumours. Pathologists and clinicians present their cases to the Registry, which re-examines the histologic slides and radiographs. From which it makes a final diagnosis and gives advice on treatment.

In 1971 the NBTR made, at our request, its files available for a retrospective study on the clinical aspects of osteosarcomas of the extremities. For the purpose of uniformity of the series some tumours were excluded:

1. parosteal or juxtacortical osteosarcomas, which have lowgrade malignancy and a good prognosis;
2. sarcomas arising in previously abnormal bone. In this respect Paget's sarcomas have almost uniformly a fatal prognosis but are fortunately extremely rare in the Netherlands as compared with the United States and England;
3. secondary sarcomas arising in previously irradiated bones.

Our series includes 176 patients which were consecutively treated between 1962 and 1968. The follow-up period was at least 3 yr; 147 cases were followed for 5 yr or more. In 1974 our study was published as a thesis [1].

Accepted 20 January 1978.

MATERIALS AND METHODS

The incidence of osteosarcomas, irrespective of its location is similar in the Netherlands to that of England, i.e. 1:400,000 annually [2].

Age and sex distribution are shown in Fig. 1. As in other large series the incidence shows a peak during the 2nd and 3rd decades. More than 68% of our patients were treated in the 2nd decade of age.

As is generally reported tumour incidence is greater in males than in females. In the youngest age group (up to 13 yr) however, the incidence in our series is relatively greater in girls (Fig. 2). The cumulative frequency of the disease in the male patients increases after the age of 15 yr, 3 yr later than in girls. In

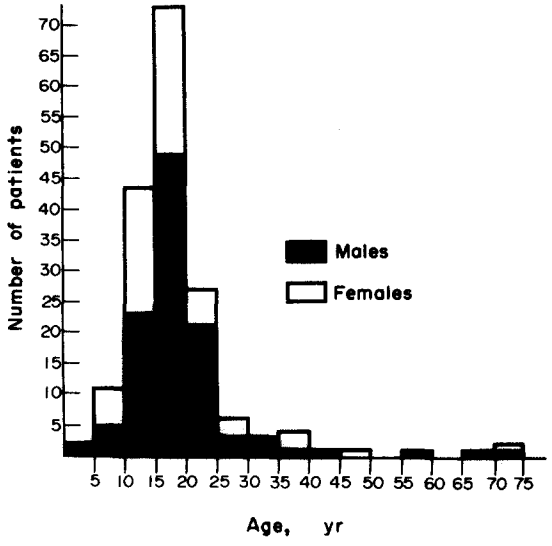

Fig. 1. Age and sex distribution of 176 patients.

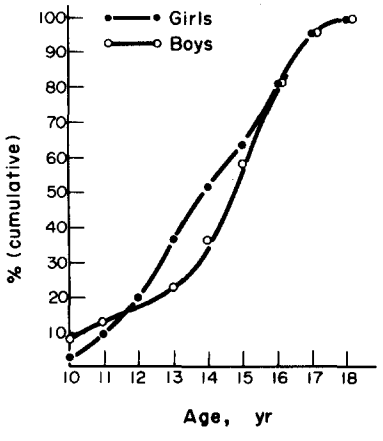

Fig. 2. Cumulative frequency of osteosarcoma in 41 girls and 62 boys between 9 and 18 yr of age.

analogy the curves representing the average increase in bodylength of boys and girls separately, and even the rate of growth of their individual long bones, show the same characteristics as the curves which represent tumour incidence according to age [1]. These differences are possibly correlated with the onset of the growth spurt during puberty. Also the many large tumour volumina in our series during and shortly after puberty may point to a correlation with a relatively large rate of growth in this period of life.

Fraumeni's conclusion [3] that children with osteosarcomas under the age of 18 yr are significantly taller as compared to those in a control group with non-skeletal tumours also points to a connection with the rate of growth during puberty.

Location

The location of the 176 tumours in our series is represented in Fig. 3. In agreement with other publications most tumours are

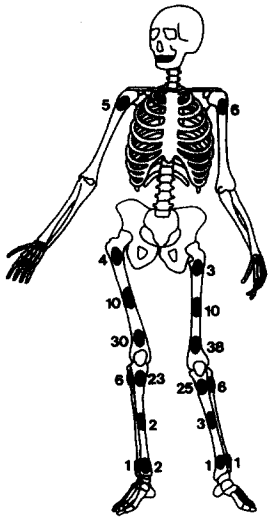

Fig. 3. Location of 176 osteosarcomas.

found in the region of the knee (65%). The primary site within the long bones is predominantly the metaphysis, sometimes extending into the shaft and in a minority of cases into the epiphysis. The numbers for right- and left-sided tumours were not significantly different.

In the distal femur the neoplasm was often found in the lateral part, whereas in the proximal metaphysis of the tibia the process was usually located in the medial part. These differences proved to be statistically significant.

Invasion into the epiphysis was extremely rare in cases with open epiphyseal plates (2 out of 57 cases in contrast to 42 out of 52 cases with closed epiphyseal plates).

Pathology

As a strict criterion for the histologic diagnosis of osteosarcoma was maintained a primary malignant tumour arising in bone which forms osteoid or bone tissue directly, i.e. without an intermediate cartilaginous stage.

The histologic slides of all cases have been critically reviewed by a small team of pathologists, all members of the NBTR. According to the dominant intercellular component a classification into osteoblastic, chondroblastic and fibroblastic types is possible, although we cannot be too emphatic in stating that with regard to the last two types such a classification is not identical clinically with chondro- and fibrosarcomas. Apart from the dominant

intercellular component we distinguish fibrosarcomatous, telangiectatic and cystic types of tumour. Fibrosarcomatous osteosarcomas were described by van der Heul [4] and are histologically characterized by a fasciculated arrangement of the tumour cells. The same author also found a more favourable prognosis for patients with such a tumour. Telangiectatic osteosarcomas are richly vascularized and contain blood filled cavities.

In accordance with the advice of the Bone Registry, in 168 cases a diagnosis was made by open biopsy, usually preceded by a short course of pre-operative irradiation within 24 hr before the biopsy (2×400 rad). In 8 patients the diagnosis was made only after a needle or drill biopsy. As to the location of the biopsy we stress the role of the clinical radiologist to guide the surgeon in the choice of the most appropriate area, i.e. the region where on the radiograph the malignant characteristics of the process are most distinctive. Only in this way can the pathologist be sure of examining a piece of tissue which represents the true nature of the tumour. This is especially important in osteosarcomas where the histologic diagnosis can be extremely difficult and even impossible when the biopsy contains only the periphery of the tumour. For that reason the biopsy should include the medulla, cortex and the surrounding soft tissue parts.

In our material 10 fibrosarcomatous and 6 telangiectatic types were found.

Radiography

As to the radiographic appearance a classification was made. We distinguish the following types:

1. a mixed osteolytic–sclerotic type (103 cases = 59%);
2. a predominantly focal sclerotic type (26 cases = 15%);
3. a predominantly osteolytic type (29 cases = 16%);
4. a cystic (7 cases) and
5. a periosteal type (10 cases).

The last two types are rare. The periosteal type was introduced by von Ronnen [5]. It is mainly characterized by spicules and with little or no changes at all in the underlying medulla. Type 1 represents the classical type and is seen in more than 50% of our cases.

In most tumours the cortical borders were completely or partially destroyed.

Periosteal changes (spicules, lamellar deposits of bone, local thickening and Codman's triangles) together with tumourous calcium deposits in the surrounding soft tissues were considered as one entity i.e. the extra-cortical reactions. These reactions lead to a great variety of radiographic characteristics, which may sometimes show very complex structures. Spicules were most often seen. Codman's triangles were apparently related to age: the older the patient the less frequently they appeared.

In our series a correct X-ray diagnosis by experienced radiologists could be made in about 2/3 of all cases. This is also mentioned in other series [6–8]. The following diagnoses were made by the radiologists of the NBTR:

X-ray diagnosis	No.	Percentage
"typical" osteosarcoma	124	70
malignant tumour, probably osteosarcoma	27	15
other malignant bone tumour	23	13
malignant bone tumour (unspecified)	2	2
Total	176	100

The differential diagnosis gave the greatest problems in cystic types of tumour, especially since the process in such cases was often initially considered as a benign one.

In our series arteriography was rarely performed. In these cases it did not provide further information concerning the malignant character of the process.

Tumour volume

In regard to the tumour size we calculated the volume roughly as follows. The process was assumed to have a cylindrical shape, the depth of which corresponded with the maximal length on the radiograph. The radius of the cross section was the average value of the maximal and minimal width as measured on both radiographs. It was not possible to make such a determination of tumour size in every case (cases with microfilms only, tumours with indistinct borders or with a pathological fracture with dislocation etc.). Tumour volume could be determined in 150 cases, being considered as small when the volume did not exceed 100 cm^3 (63 cases = 42%) and large where a volume of 200 cm^3 or more was found (41 cases = 27%). The number of medium sized tumours (100–200 cm^3) was 46 (31%). Large tumours were especially found in the

femur, the smallest in the capitulum fibulae. As we mentioned earlier the relatively great number of large tumour volumina during and shortly after puberty was striking (50%, in contrast to 25% for patients older than 20 yr).

Symptomatology and clinical examination

The average duration of the interval between onset of symptoms and treatment was $3\frac{1}{2}$ months. In 65% (111 patients) therapy was started within 3 months. Especially in the very young children symptoms were as a rule present for a few weeks only. In 23 patients first treatment was inadequate because the process was at first considered to be a benign condition such as osteomyelitis, arthritis or a cyst. No correlation between tumour size and duration of symptoms was found. Trauma was mentioned with 51 patients (28%).

The symptoms most often seen were pain and swelling. In only a few cases swelling preceded the pain. Pain was absent from 21 patients. Physical examination revealed the following findings:

1. swelling of the bone and/or soft tissues which usually was tender;
2. discoloration of the skin, sometimes with hypervascularization;
3. impairment of the normal function of the joint, sometimes with contracture;
4. muscular atrophy, especially of the thigh in cases of a location near the knee;
5. paresis of the peroneal nerve in tumours occurring in the proximal end of the fibula.

The blood sedimentation rate before biopsy was performed in 99 patients. In 29 cases only elevated values were found. In 95 patients the blood alkaline phosphatase was determined before treatment. In only 26 of these 95 cases was the determination repeated at regular intervals during and after treatment. It was found that in 52 patients (54%) the initial values were increased i.e. exceeding the values regarded in the particular hospital laboratory as normal for the age of the patient. In regard to tumour volume we found a positive correlation between increased alkaline phosphatase levels and tumour volume (Table 1), which indicates that in osteosarcoma quantitative factors may be of greater influence on the alkaline phosphatase activity than qualitative characteristics of the tumour. In all cases an increase of the alkaline phosphatase during follow-up was associated with a rapid clinical manifestation of pulmonary metastases, although there was in some cases local tumour activity after radiotherapy as well. In 10 patients a pathological fracture, most frequently in the humerus, was found before a final diagnosis was made. Telangiectatic tumours which clinically were characterized by hypervascularization with redness and elevated temperature of the overlying skin, lead in some cases to the erroneous diagnosis of an osteomyelitis.

Metastatic patterns

The main factor in the course of the disease is the occurrence of pulmonary metastases. In our series these metastases occurred in over 78% of patients. During a follow-up period of more than 3 yr only 38 of 176 patients (22%) remained free from pulmonary metastases. In 5 cases (3%) these metastases were already present at the time of diagnosis.

The median time for a clinical metastases-free interval was 8 months from the start of treatment. In our series 40% of all patients developed lung metastases within 6 months and 60% within one year (Fig. 4).

In 86 chest X-rays, which showed the metastases for the first time the location was found to be unilateral in more than 50%. In most cases the number of these metastases was initially limited. In 13 patients tumourspread to the lungs was multiple and diffuse from the beginning.

Most metastases were dense with rather sharp borders, the configuration being round or oval.

Table 1. Correlation tumour volume and alkaline phosphatase

Alkaline phosphatase	Tumour volume					
	< 100 cc		100–200 cc		> 200 cc	
	No.	%	No.	%	No.	%
Increased	15	42	12	54	17	71
Normal	21	58	10	46	7	29
Total	36	100	22	100	24	100

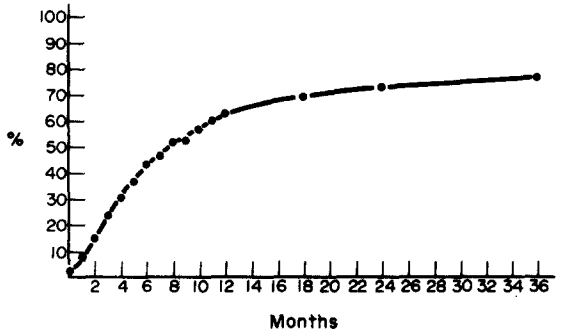

Fig. 4. The cumulative frequency of pulmonary metastases in 176 patients.

Calcification of metastases may occur, sometimes at an early stage, combined with a decrease in growth rate or even arrest of growth. Such a phenomenon was seen in 10 cases. Calcification of miliary metastases was observed in 5 out of 6 of these cases within 4 months. In general, calcification of solitary secondaries occurred at a later stage, although always within 10 months. Except in one of the 10 patients all cases presented a tumour which was radiologically of a sclerotic type. In the beginning pulmonary metastases produce few symptoms, but may be suspected where cough, fever and dyspnoe occur. Haemoptysis is rare. Cytology of the sputum is usually tumour negative. In exceptional cases a superior vena cava obstruction or intercostal neuralgia was found. We saw 3 cases with a hyperplastic osteoarthritis. Pleurisy usually is a terminal complication. A pneumothorax is not uncommon (12 cases). In one patient mediastinal emphysema developed a few days before death.

In 26 patients it was possible to measure the increase in diameter of 49 lung metastases after a known interval. Measurements were performed as described by Collins [9] and Breur [10]. Plotting the logarithm of the diameter as a function of time on a linear scale the growth curves obtained corresponded with a straight line. Based on this line we calculated the volume doubling time (T_d) of each metastasis separately.

Apparently these secondaries have a constant rate of growth during the stage of their clinical development. Variations between the growth rate are shown in Table 2. The mean doubling time was 38 days. Except for a few cases measurements in the same patient did not result in significant differences of the T_d. Survival time after the diagnosis of pulmonary metastases decreased with increasing growth rates (Table 3). In all cases extrapolation of the growth curves lead to the conclusion that these metastases existed already at the moment that treatment was started. Data on 8 pulmonary metastases in 5 patients during and after radiotherapy of these foci gave evidence of an exponential decreasing curve, the gradient of which was steeper than the original growth curve before treatment. In one case spontaneous disappearance of one metastasis also occurred after a period of exponential decrease. The frequency of osseous metastases was about 13% (24 out of 176 cases).

Three patients with skeletal metastases died without clinically detectable lung metastases. In 7 of 21 patients the detection of the skeletal and pulmonary metastases took place at the same time. In 2 patients the osseous metastases manifested themselves one to two months before the detection of the pulmonary secondaries. In 14 cases however, pulmonary metastases were already present. As a rule the period between the detection of the pulmonary and skeletal metastases was relatively

Table 2. Doubling times in days of 49 pulmonary metastases in 26 patients

	10–20	20–40	40–80	80–160	>160
Number of metastases	5	19	22	2	1

Table 3. Doubling time of pulmonary metastases and survival

Doubling time in days	No. patients	Mean survival in months	Median survival in months
0–20	2	8	—
20–40	12	14.6	12
40–80	10	18.9	16.5
>80	2	43	—

short (up to 2 months). The radiological aspect of the osseous metastases was identical to that of the primary tumour.

Clinical symptoms of cerebral metastases developed in 8 patients. Histologic confirmation was possible in 3 cases. All these patients succumbed within 2 months.

Other locations of tumour spread in our series were the subcutaneous tissue (3 cases), kidneys and peritoneal cavity (6 cases).

The literature as well as our own data give unsatisfactory information on the frequency of metastatic involvement of the regional lymph nodes. Most publications mention percentages based on post-mortems and on examination of surgical specimens. Systematic lymphography in osteosarcomas points to a relatively high incidence of lymphatic involvement as compared to other malignant bone tumours [11]. In our material metastatic lymph nodes were found on physical examination in 8 cases only.

Treatment

From 1954 until 1974 the NBTR has advised, as the treatment of choice, primary radiotherapy followed where indicated by ablative surgery at a later time, following the same line of reasoning as advocated by Cade [12]. In accordance with this policy the therapeutic measures taken were as follows.

1. After histologic confirmation of the diagnosis the tumour was irradiated to a tumour dose of 6000–7000 rad in 5–6 weeks (128 cases);
2. Ablative surgery was only performed when after an observation period of 4–6 months no clinical evidence of metastases was found (65 cases).

In the absence of metastases immediate radical surgery was done in:

1. cases where according to histologic or radiographic criteria a more favourable prognosis was to be expected (fibrosarcomatous types, tumours with a low mitotic index, periosteal and cystic forms) (15 cases);
2. cases with a pathological fracture or with serious impairment of normal function or when the skin was infiltrated by the tumour; (7 cases);
3. cases in which for some time inadequate treatment had been given because a non-malignant condition was assumed to be present (3 cases).

The advice of the NBTR was followed by most of the clinicians. A few surgeons however, preferred amputation for all their cases (17 patients).

Table 4 gives a survey of the methods of treatment used in our cases. In 5 cases treatment of the primary tumour was palliative from the beginning since pulmonary metastases were already present at the time of the first examination. In one case the available data were incomplete. In 128 patients (75%) treatment was started with radiotherapy. In this group 65 amputations were performed after an observation period, as no metastases had occurred. 63 other patients developed pulmonary metastases within the post-irradiation observation period. In 9 cases a palliative amputation had to be done in spite of metastases. Primary amputation was performed in 42 patients. In accordance with other publications [13–15] microscopy of the amputated irradiated limbs showed viable tumour tissue in about two-thirds of the specimens. The palliative effect of radiotherapy on the local symptoms was variable and unpredictable. In a group of 80 patients with a well documented history during the first months after radiotherapy we found clinical evidence of recurrent tumour growth in more than 50% (43 cases). Especially in those patients where pulmonary metastases manifested themselves at an early stage, complete tumour remission was the less frequent, meaning that in cases where no curative ablative surgery

Table 4. Therapy of 170 patients with osteosarcoma

Therapy	No.	%
Radiotherapy only	54	32
Radiotherapy + ablative surgery (curative)	65	38
Radiotherapy + palliative amputation	9	5
Primary amputation	42	25
Total	170	100

was indicated the local response after irradiation was the less favourable. We estimate that at the time of death the tumour in non-operated cases was active in about 75%. In agreement with the experience of Jenkin [16] we conclude that preoperative irradiation has failed in its role of providing adequate local palliation.

RESULTS

Survival in both sexes is identical. The gradient of the survival curve is steepest between 4 and 12 months after starting treatment (Fig. 5). Within a year mortality in our series was about 40%. The 3- and 5 yr survival rates are 53/176 (30%) and 33/148 (22%) respectively.

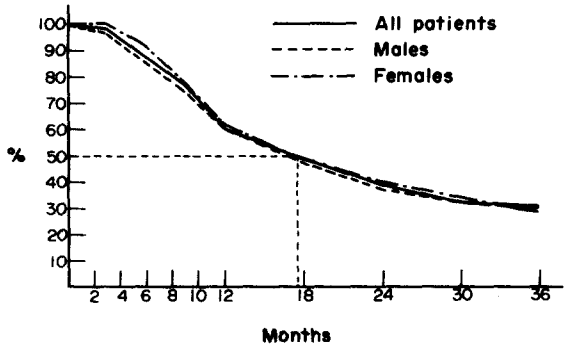

Fig. 5. Survival curves of 176 patients (111 males and 65 females).

Considerable differences in survival can only be shown for selected groups of patients. For instance, survival will greatly improve after primary radiotherapy when an amputation is performed after a metastases-free interval (Fig. 6). The results of a primary

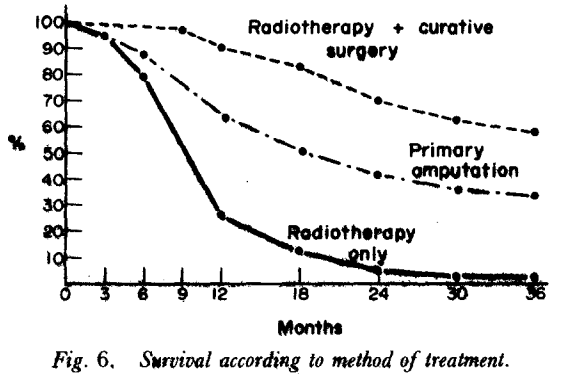

Fig. 6. Survival according to method of treatment.

amputation are influenced by selection of cases with favourable prognostic criteria.

In our series survival after primary radiotherapy in a group of 128 patients is as follows:

Primary radiotherapy		
	3-yr-survival	5-yr survival
Total series	39/128 = 30%	23/105 = 22%
Radiotherapy only	2/63 = 3%	1/49 = 2%
Radiotherapy + planned delayed amputation	37/65 = 57%	22/56 = 39%

From these results it becomes clear that radiotherapy followed by operation after an observation period results in a selection of patients for which prognosis is significantly more favourable. The survival rates after primary ablative surgery are:

Primary surgery		
	3-yr survival	5-yr survival
Total series	14/42 = 33%	10/38 = 26%
Patients with favourable prognostic criteria	8/15 = 53%	6/12 = 50%
Other cases	6/27 = 22%	4/26 = 15%

In these 42 patients differences in indication for treatment have also lead to differences in therapeutic results.

Prognosis

No differences in prognosis were found as to age, sex and tumour site. For patients with a histologic fibrosarcomatous type of tumour, as described by van der Heul [4], prognosis was more favourable. Six of 10 cases with such a tumour are alive, more than 5 yr after the beginning of treatment. A low number of mitoses, i.e. 1–2 mitoses as seen in more than one microscopic field, magnification 500–700 ×, also corresponded with a better prognosis. For these patients a 3-yr survival of 52% and a 5-yr rate of 33% were found. For that reason the advice of the NBTR to perform a direct amputation has retrospectively been justified. The volume of the tumour appeared to have no relation with prognosis.

In accordance with the opinion of von Ronnen [5] the radiological cystic and periosteal types have a more favourable prognosis. In 4 of 7 cystic types, however, the number of mitoses was also low. Furthermore 2 of these 4 tumours could be classified as

fibrosarcomatous as well. The 5-yr survival rates of the cystic and periosteal types are 6/7 and 4/10 respectively. Calculation of the survival rates for tumours classified according to various amounts of spicules and lamellar periosteal bone deposits showed surprisingly a positive correlation with prognosis. We started from the observation that a large number of patients with a survival of 5 yr and more had suffered from a primary tumour with relatively many periosteal spicules and/or lamellar bone deposits.

The percentage of these periosteal reactions was calculated as follows. On the a.p. and lateral radiograph of the tumour we measured along every border separately the total length (l_i) of the spicules and lamellar deposits as well as the total length (L_i) of all visible extra-cortical reactions. All l-values were added up, divided by the sum of all L-values and multiplied by 100. Figure 7 gives an imaginary example.

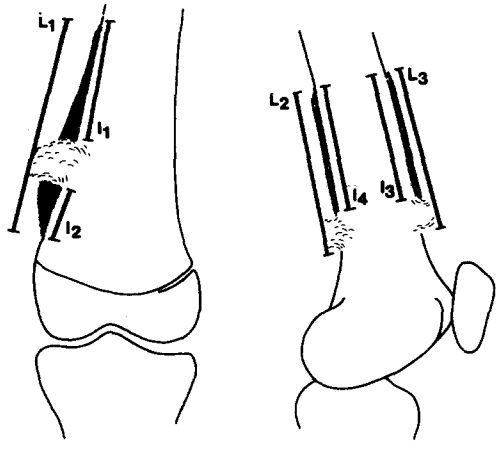

Fig. 7. Calculation of percentage spicules and lamellar bone deposits in relation to the tumour surface (see text). Percentage is:

$$\frac{l_1+l_2+l_3+l_4}{L_1+L_2+L_3} \times 100.$$

Based upon the percentage spicules and lamellar deposits a classification into 4 groups was made (Table 5). As can be seen prognosis improves in proportion to the increase of the amount of spicules and lamellar deposits.

Table 5. Survival related to the amount of spicules and/or lamellar periosteal bone deposits

Percentage	3-yr survival		5-yr survival	
	No.	%	No.	%
Till 25%	5/33	15	3/32	9
25–50%	7/19	37	3/17	18
50–100%	13/30	43	9/22	40
100%	10/15	66	8/13	61

Differences in survival rate proved to be statistically significant.

These findings may be explained by differences in the rate of growth as an exponent of the grade of malignancy of the process. Rapidly growing tumours prevent periosteal formations with a more or less regular structure. This hypothesis is supported by the observation that in some cases the rate of growth of the tumour is slowed down by radiotherapy in such a way that spicules occur in an area where those structures originally were absent. We propose to refer to tumours covered for more than 50% with spicules and/or lamellar bone as "structural tumours".

In our series this relates to 45 patients (25% of the total group). From what has been said in regard to prognosis it follows that primary treatment for these structural tumours should be primary ablative surgery.

Naturally survival is determined to a great extent by the interval between start of treatment and appearance of the first pulmonary metastases

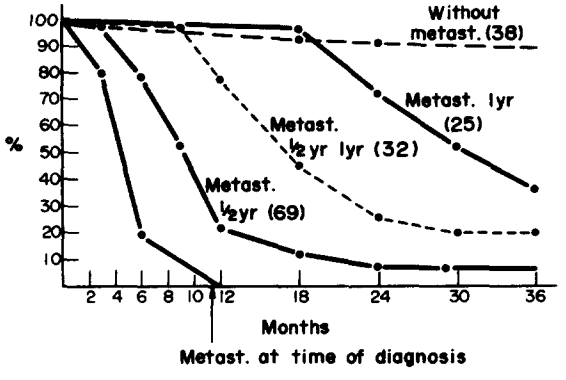

Fig. 8. Correlation 3-yr survival rate and time of detection of lung metastases. Note differences in steepness of curves.

(Fig. 8). We want to point out that the longer the metastases-free interval the less steep the survival curves. The course of the disease during the stage of lung metastases is prolonged in proportion to the later manifestation of these metastases.

DISCUSSION

From what has gone before it appeared (as was also shown by other authors) that some histologic and radiographic criteria often go together with a more favourable prognosis. We found that this also holds true for a new criterion, i.e. the structural tumours as was discussed earlier. In many cases however, there is a combination of different criteria, leading to a considerable overlapping of such prognostic features. Further analysis has lead

us to a classification into two groups. The first group is made up of tumours with one or more histologic and/or radiographic criteria correlated with better prognosis. This group composes in our series 69 patients.

The second group is formed by 106 patients suffering from osteosarcomas without such criteria. The 3-yr survival rate of group I is 52%, whilst that of group II is only 18%. Group I was subdivided into smaller units which show the 3-yr survival for the various prognostic features separately (Fig. 9). As was described

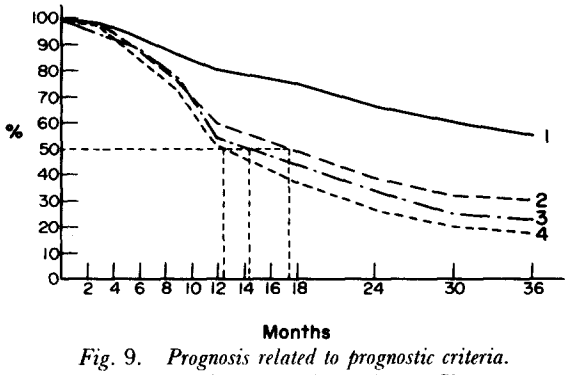

Fig. 9. Prognosis related to prognostic criteria.
1. 56 *patients with structural, cystic or fibrosarcomatous tumours;*
2. *all* 176 *patients;*
3. 52 *patients with tumours characterized by spicules and lamellar bone less than* 50% *of the total tumour surface;*
4. 129 *patients without prognostically favourable characteristics.*

previously, in the majority of cases treatment was started by radiotherapy. Taking into account the differences in indication for the various treatment schedules, the type of treatment, as long as aimed to be "radical", had no influence on prognosis.

Our own experiences and some new developments however, have caused doubts on the effectiveness of preoperative radiotherapy as the treatment of choice. First of all the "Cade" principle of radiotherapy and a post-irradiation observation period had been chosen to save the emotional stress of an amputation for about 75% of all patients in which ablative surgery does not add to the chance of a complete cure. All emotional and psychologic aspects of radiotherapy as a means of selection of patients where amputation is indictated at a later time are practically unknown until now. Furthermore the palliative local response of irradiation has proved itself insufficient in many cases. Jenkin [16] came to exactly the same conclusion, whereas Cade [12] pointed to the fact that amputation after radiotherapy was performed mainly for palliative reasons in 44 of 76 patients. The need for a delay of the amputation is far less for the group of patients with a prognostically more favourable tumour. In our series classification into structural tumours has enlarged the indication of a primary amputation to 32%.

Finally the results of elective irradiation of the lungs [17] and prophylactic chemotherapy [18–20] have also given a new dimension to a less fatal outcome of this tumour process. These elective treatments seem to be able to reduce the clinical manifestation of metastases considerably (about half of the total group). This will also diminish the urgency for a selection through an observation period.

As to the osseous metastases the curve representing the cumulative percentage in relation to the interval in months after treatment (Fig. 10) is practically identical to a

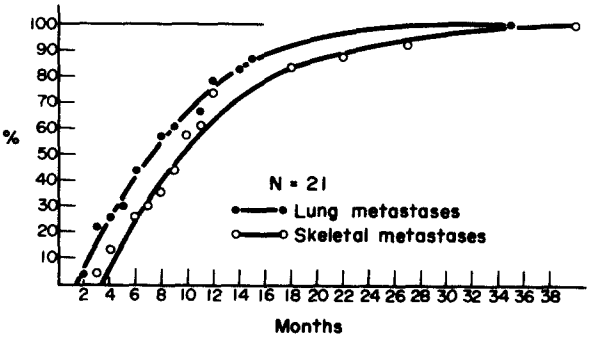

Fig. 10. The cumulative frequency of lung and skeletal metastases.

similar curve of the pulmonary metastases with a relative shift of about 2 months. This may suggest that most of the osseous metastases are true secondaries, similar to the lung metastases. The average short period between the clinical detection of the pulmonary and osseous metastases makes it very unlikely that a longer survival by prophylactic treatment will increase the number of patients in which osseous metastases will become manifest.

For cases where primary radiotherapy still remains indicated the possibilities of new radiation techniques should be examined such as fast heavy particles and combinations with hypoxic sensitizers.

In short we have come to the following therapeutic scheme for peripheral osteosarcomas which has been followed by the NBTR since 1975.

1. Ablative surgery for the primary tumour followed by elective treatment (irradiation of the lungs or chemotherapy) in all cases without detectable metastases. In particular radical surgery is strongly indicated for tumours fulfilling prognostic favourable criteria or in cases with a pathological fracture or

serious malfunction of the extremity. 2. Initial irradiation with a high dose of the primary tumour has to be considered in cases where amputation is refused. Elective treatment should also be considered. A proposition for amputation should be repeated afterwards. 3. For patients with metastases a combined treatment of radiotherapy and chemotherapy should be given.

All patients whose condition requires ablative surgery should be given expert guidance from the moment he comes in for treatment until he has mentally and physically adapted himself to his handicap. This is a subject which urgently requires further study. We are of the opinion that for an optimal care patients with osteosarcomas should be treated in oncological centres.

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Effects of Antimitotic Agents Bound to a Macromolecular Carrier in Normal or Cancer Cells. Importance of the Mode of Binding of the Agent to the Carrier*

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Abstract—*Normal or cancer cells have been treated* in vitro *or in the animal with* cis-*dichloro-diammine platinum (II)* (cis-*Pt*), *a potent antimitotic agent. When the latter is covalently linked to a macromolecular carrier (DNA), or even to a mononucleoside (guanosine or adenosine) it loses its antimitotic activity. On the contrary, in the case of ethidium bromide (EB), another antimitotic agent, the cytotoxicity for tumour cells is maintained or even enhanced after binding (by intercalation) to DNA.*

INTRODUCTION

THE ANTITUMOUR efficiency of a chemical agent can be enhanced when it is bound to a macromolecular carrier. Trouet *et al.* [1, 2] have treated with success, murine or human leukemias with daunorubicin or adriamycin bound to high molecular weight DNA. We have demonstrated that ethidium bromide (EB) bound to DNA is more cytotoxic to Ehrlich ascites tumour cells than free EB [3, 4]. It is well known [1–4] that the complex antimitotic agent-macromolecular vector is absorbed by the cells; the agent is then released from the vector, which is digested by lysosomal enzymes, and diffuses in the cell. Cells with a high endocytic activity can absorb such complexes; various tumour cells do so. During this "lysosomal therapy", normal cells with low endocytic activity can be somewhat "protected" against the antimitotic agent. For example, EB–DNA added to the culture medium of chick fibroblasts provokes less cytological alterations than free EB does [5].

The mode of binding of the agent to the carrier is of great importance. The complex antimitotic agent-carrier must be stable in the extracellular medium but the agent must be liberated inside the cells. Up to now, daunomycin, adriamycin and ethidium bromide have been used after binding to DNA. These agents bind to DNA by intercalation between adjacent base pairs. This binding is stable in the extracellular medium but can be destroyed in the cells [1, 5]. We thought it was interesting to treat cells with a complex in which the cytotoxic agent is bound to DNA, not by intercalation, but covalently. We have chosen *cis*-dichlorodiammine platinum (II) (*cis*-Pt), discovered by Rosenberg and coll. [6]. This agent covalently binds to guanine bases [7]. *Cis*-Pt has a strong antimitotic activity in normal or cancer cells [8–11]. In the present study, we have treated normal or tumour cells with *cis*-Pt bound to DNA or to a nucleoside (guanosine or adenosine).

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MATERIALS AND METHODS

Chick fibroblasts freshly prepared from embryos were cultivated in plastic Falcon dishes, in a liquid medium: 90% Eagle's minimum

medium, 10% foetal calf serum and 100 U penicillin per ml.

Mouse peritoneal macrophages were collected from the peritoneal cavity of C57 Bl. mice by washing with an isotonic Hanks' solution and then cultivated (in Falcon dishes) in a liquid medium: 45% NCTC 109 medium, 50% Hanks' solution, 5% foetal calf serum and 100 U penicillin per ml.

Ehrlich ascites tumour cells (tetraploid line) were collected from C57 Bl. mice bearing the tumour. The cells were then cultivated (in Falcon dishes) in a liquid medium: 45% NCTC 109 medium, 45% Hanks' solution, 10% foetal calf serum and 100 U penicillin per ml. The culture medium was renewed every 24 hr in order to maintain active cell proliferation.

Treatment by cis-*Pt*. *Cis*-Pt, free or bound to DNA, guanosine or adenosine was added to the culture medium at the final concentration of 5 μg/ml or injected into the peritoneal cavity of mice bearing Ehrlich tumour cells at the doses of 4.5 mg/kg fresh weight.

Preparation of the complexes. *Cis*-dichlorodiammine platinum (II) (Polysciences Inc.; NSC 119875; 200 μg/ml) was mixed with DNA (Sigma, PM $\pm 10^6$, calf thymus, 1 mg/ml in $NaClO_4$ 0.01 M) and NaCl (0.1 mM) at 37°C and in darkness. The reaction lasted 20 hr; only 2% *cis*-Pt or less remain free. Thereafter NaCl concentration was increased up to 0.1 M. The ratio of *cis*-Pt to DNA was of 0.20 to 0.25.

Cis-Pt-nucleoside complexes were prepared in a similar way: 200 μg *cis*-Pt per ml were mixed with guanosine (Schwartz Laboratories: 378 μg/ml) or adenosine (Schwartz Laboratories: 534 μg/ml).

The mitotic activity was estimated by calculating the mitotic index (number of mitotic cells for 1000 cells). In each case, 2000–3000 cells have been observed.

The number and the volume of the Ehrlich tumour cells was calculated with an electronic Coulter counter (Model B).

The DNA content was measured in individual cells by absorption cytophotometry after Feulgen reaction (hydrolysis by HCl 3.5 N, 20 min at 37°C) with a GN2 scanning microdensitometer from Barr and Stroud, at 540 nm.

The total protein content was measured in individual cells by cytophotometry after naphtol yellow S staining with a GN2 microdensitometer from Barr and Stroud at 435 nm.

The incorporation of tritiated guanosine, free or linked to *cis*-Pt (specific activity: 10 μCi/mM; final concentration: 10 μCi/ml) was analysed by histoautoradiography. After 30 or 60 min of contact, the cells were rinsed in an isotonic saline solution and fixed with absolute ethanol/acetone (1/1). The liquid emulsion method was applied (L4 Ilford emulsion, Microdol X as developer).

Electron microscope observations. Cells were fixed with 2.5% glutaraldehyde and postfixed with osmium tetroxide (1%). Inclusion was performed in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate. The ultrastructural observations were realized with a Siemens Elmiskop 101 at 80 kV.

RESULTS

Effects of free cis-*Pt*

As we have shown previously, when chick fibroblasts cultivated *in vitro* or Ehrlich ascites tumour cells cultivated *in vitro* or propagated in the mouse are treated with *cis*-Pt, nucleolar and chromatin alterations are observed. The mitotic activity is inhibited (Figs. 1 and 4) and many cells are blocked either in the G1 or the G2 phase of the cycle (Fig. 2) according to the concentration that is used. Moreover, *cis*-Pt can provoke a considerable increase of the cell volume, because RNA and protein synthesis remain active (Table 1). Many abnormal mitotic figures appear in the treated cells (for more details, see [10, 11]).

Table 1. *Mean protein content measured by cytophotometry after naphtol yellow S staining in* cis-*Pt* (4.5 mg/kg) *or* cis-*Pt-DNA* (cis-*Pt:* 4.5 mg/kg) *treated Ehrlich tumour cells and in controls* (NaCl:0.1 M *solution*)

Controls	45.16 ± 0.92
Cis-Pt	81.92 ± 1.59
Cis-Pt-DNA	44.92 ± 1.45

The cells have been collected 6 days after the intraperitoneal injection of the solution. These mean values are expressed in arbitrary units and have been calculated from results obtained for 50 cells in each case. The standard error is indicated.

Cis-Pt (5 μg/ml) provokes nearly no alterations in mouse peritoneal macrophages cultivated *in vitro*: their cytological structure remains normal and their endocytic activity is not diminished (see also [12]).

Effects of cis-*Pt bound to DNA*

(a) When chick embryo fibroblasts or Ehrlich tumour cells are cultivated in the

presence of *cis*-Pt-DNA complex (*cis*-Pt: 5 μg/ml), no cellular alterations can be detected, even after 3 days. The fibroblasts (Fig. 1) and the tumour cells keep a normal mitotic activity. Moreover, measurements of the DNA content performed in these treated cells (fibroblasts or tumour cells) show no differences as compared to control cells.

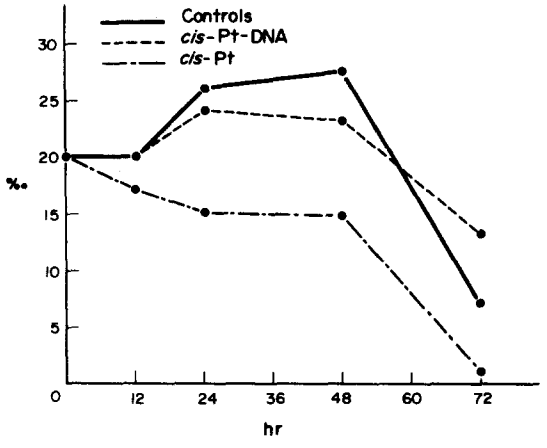

Fig. 1. Mitotic activity in cis-*Pt* (1 μg/ml) *or* cis-*Pt-DNA* (cis-*PT:* 1μg/ml) *treated fibroblasts cultivated* in vitro. *In abscissae: duration of the treatment; in ordinates: number of mitoses for* 1000 *cells.*

(b) Ehrlich ascites tumour cells were treated with *cis*-Pt-DNA (*cis*-Pt: 4.5 mg/kg) in the mouse peritoneal cavity. Their mitotic activity (index: 29–30‰) remains similar to that of the controls (24–35‰); their DNA content and their total protein content is not modified (Fig. 2 and Table 1). On the contrary, *cis*-Pt treated cells (4.5 mg/kg) are blocked in the G2 phase (Fig. 2) and their total protein content is strongly increased (Table 1). This is due to the fact that protein synthesis remains active whereas cell division no longer occurs.

(c) Mouse peritoneal macrophages treated *in vitro* with *cis*-Pt-DNA (*cis*-Pt: 5 μg/ml) have a normal morphology and are able to phagocyte latex particles at the same rate as the controls do, even after 72 hr of treatment.

After a treatment with *cis*-Pt-DNA, fibroblasts, macrophages and tumour cells contain cytoplasmic vacuoles filled with a dense material (electron microscope observations). These vacuoles are particularly numerous in macrophages treated *in vitro* with the complex (Fig. 3). We have shown with an electron microprobe that these vacuoles contain platinum. We think that these vacuoles (probably phagolysosomes) are filled with *cis*-Pt-DNA (see also [13]). *Cis*-Pt linked to DNA is thus absorbed by the cells but cannot exert any cytotoxic action.

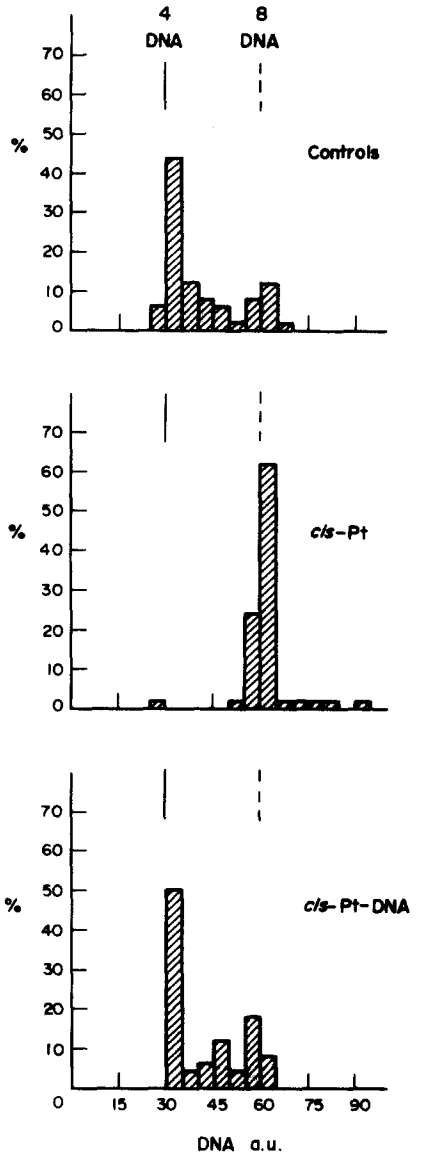

Fig. 2. DNA content measured by absorption cytophotometry in Ehrlich tumour cells treated with cis-*Pt* (4.5 mg/kg), *with* cis-*Pt-DNA* (cis-*Pt:* 4.5 mg/kg) *or with* 0.1 M NaCl (*controls*). *The tumour cells have been collected* 4 days *after the injection of the solution into the peritoneal cavity of mice bearing the tumour. In abscissae: DNA content in arbitrary units; in ordinates: percentages of cells in each class. Full lines at the top of the histograms indicate the mean DNA content measured in post-mitotic cells (tetraploid line); this value has been doubled (dotted lines).*

Effects of cis-*Pt bound to guanosine or to adenosine*

We have also treated cells (fibroblasts, macrophages or tumour cells) with *cis*-Pt bound to a mononucleoside (guanosine or adenosine). The aim of this experiment is to try to find out whether mononucleosides bound to *cis*-Pt which probably appear in the cell after depolymerisation of the complex *cis*-PT-DNA, are cytotoxic.

Tritiated guanosine bound to *cis*-Pt penetrates into cells (Ehrlich tumour cells and

macrophages): it can be detected by histoautoradiography in the cytoplasm and in the nucleus, mainly in the nucleoli.

When Ehrlich tumour cells are treated *in vitro* or *in vivo* with *cis*-Pt bound to guanosine (5 μg *cis*-Pt/ml in the culture medium or 4.5 mg *cis*-Pt per kg, by intraperitoneal injection), no morphological cellular injuries are detected and cell multiplication is unaffected: the mitotic index remains normal and results of DNA measurements show no differences as compared to the controls. When *cis*-Pt (4.5 mg/kg) bound to adenosine is injected into the peritoneal cavity of mice bearing an Ehrlich ascites tumour, the number of tumour cells is decreased a little, 2 and 4 days after the injection (Fig. 4), but after 6 days, it is at the same level as in control mice. Adenosine alone has no effect, but free *cis*-Pt strongly inhibits cell multiplication.

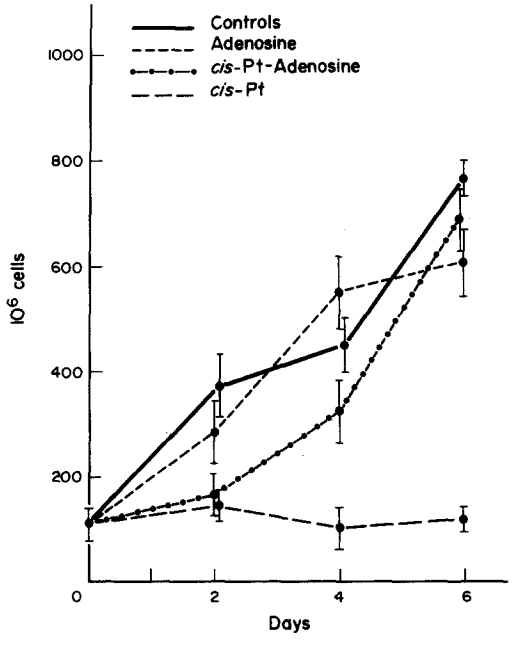

Fig. 4. Number of Ehrlich tumour cells, measured with an electronic Coulter counter, in mice treated with cis-*Pt* (4.5 mg/kg), cis-*Pt-adenosine* (cis-*Pt*: 4.5 mg/kg), *adenosine* (5.3 mg/kg) *or* 0.1 M NaCl (*controls*). *Each value is a mean calculated for* 8 *mice in each case. The standard error is indicated.*

DISCUSSION AND CONCLUSIONS

Cis-Pt is a cytotoxic agent, able to inhibit the mitotic activity and to kill cells [8–11]. In the present study, we show that, when cis-Pt is bound to DNA, it can penetrate into the cells (fibroblasts, macrophages, Ehrlich tumour cells) and accumulated inside cytoplasmic vacuoles, however this complex is nearly not cytotoxic. This loss of cytotoxicity seems to be related to the mode of linking of *cis*-Pt to DNA. In collaboration with C. Houssier and M. C. Gillet [14], we have demonstrated that lysosomal enzymes (extracted from rat liver), acid DNase and neutral DNase (deoxyribonuclease, Worthington) cannot liberate *cis*-Pt from DNA when the complex is incubated *in vitro* with these enzymes. The binding of *cis*-Pt to guanine base [7] is probably too strong. This interpretation is confirmed by our experiments concerning the effects of *cis*-Pt bound to nucleosides: when *cis*-Pt is bound to guanosine (this binding is very strong: the formation constant is about $10^9 M^{-1}$ [15]), no cytotoxic effects can be detected in the treated cells. However, this complex *cis*-Pt-guanosine penetrates into the cells, as we have shown by histoautoradiography. When *cis*-Pt is bound to adenosine (this binding is less strong than in the case of guanosine; the formation constant is about $10^5 M^{-1}$ [15]), some cytotoxic effects are observed; these could be due to some free *cis*-Pt present in the solution of the complex.

Other agents used after binding to DNA (adriamycin, daunorubicin, ethidium bromide, see Introduction) intercalate between base pairs in DNA; as soon as DNA is digested inside the cell, the drug is liberated. It seems that lysosomal hydrolases play an important role in this liberation [1]. We have demonstrated that ethidium bromide–DNA complex penetrates into the cells by endocytosis and accumulates in cytoplasmic vacuoles. EB is liberated and diffuses in the cell, reaching the nucleus and the mitochondria [5]. But the mode of binding of the agent to the vector is not the unique important factor. The cells must be able to endocyte actively the complex and to digest the carrier. In fact, in cells with a low endocytic activity, the free drug may act more intensively than the same drug bound to a carrier. We have observed this fact in chick fibroblasts cultivated in the presence of ethidium bromide [5]. In general, tumour cells have a relatively high endocytic activity; this can explain why daunomycin, adriamycin or ethidium bromide when bound to DNA are very cytotoxic for these cells [1, 2, 4]. But cells of the histiocytic system also have a very high endocytic activity and can thus absorb a great quantity of such complexes. Our observations related to ethidium bromide–DNA treated mouse peritoneal macrophages have in fact shown that these cells are very sensitive to this treatment [12].

The different clearance pattern between

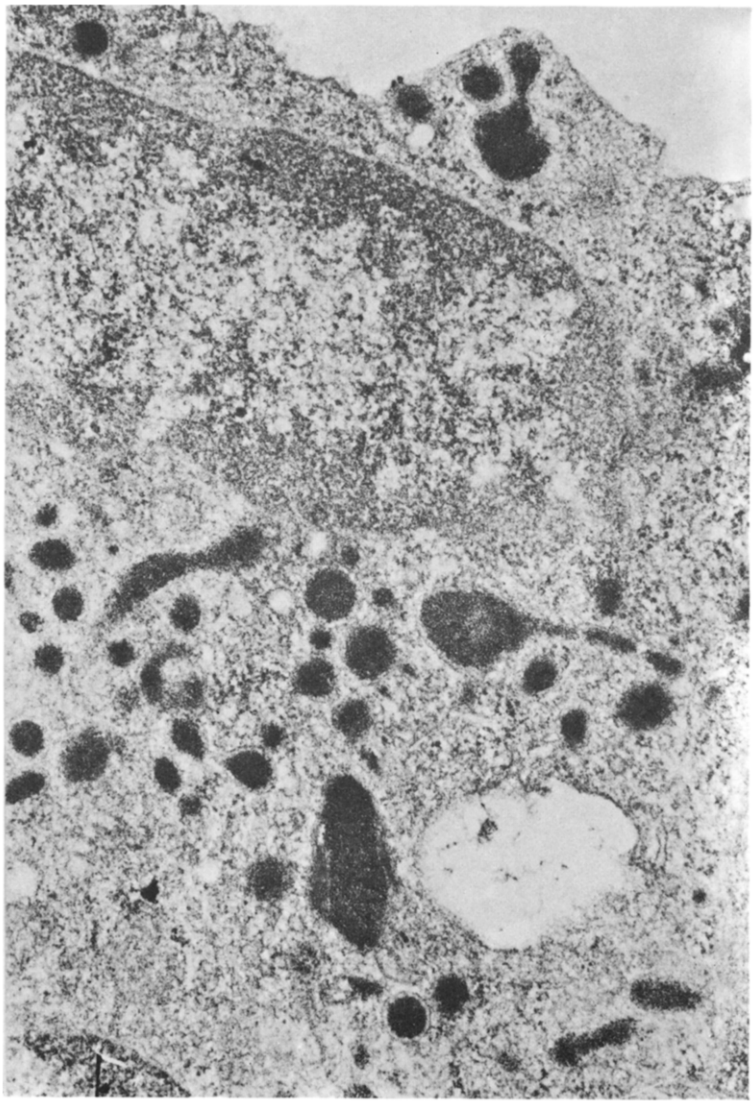

Fig. 3. Portion of a mouse peritoneal macrophage cultivated in vitro *for* 72 hr *in the presence of* cis-*Pt-DNA* (cis-*Pt:* 5 μg/ml). *In the cytoplasm, several vacuoles containing an electron dense material are visible* (*magn.* 33,000 ×).

free or bound drugs is another factor which has to be considered. In a previous study [4], we have demonstrated that EB bound to DNA persists after intraperitoneal infection for a longer time in the peritoneal cavity than free EB does. The ascites tumour cells are thus in contact for a longer period. This has been confirmed by fluorescence microscopy observations showing that EB–DNA treated tumour cells remain fluorescent during more hours than free EB treated tumour cells do [4]. Rozencweig *et al.* [16] drew similar conclusions concerning adriamycin and DNA–adriamycin complex injected to patients or mice, with regard to initial plasma levels and patterns of clearance from the plasma.

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Characterization of Methotrexate Recovered from the Urine of High Dose Patients for the Purpose of Clinical Re-Use*

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Abstract—*High dose methotrexate (MTX) therapy with leucovorin rescue is now frequently used in cancer chemotherapy especially in the adjuvant treatment of osteogenic sarcoma. Due to the large quantities of MTX required the cost of this therapy has limited its use in many European oncology centers. Since as much as* 90% *of the MTX dose is excreted largely unmetabolized in the urine during the first* 24 hr *following administration, the possibility of the recovery of the drug for re-use was investigated. From the urine of patients having received* 10–18 g *MTX, at least* 50% *of the initial dose was recovered for clinical re-utilization. MTX was purified from urine primarily by acid precipitation. Calcium phosphate treatment and ultrafiltration in the purification procedure were used for the removal of pyrogens. The urine-derived drug preparation was* 90–95% *MTX based on absorbance at* 260 nm *after fractionation on DEAE cellulose and contained impurities with spectral properties characteristic of pteridine compounds as found in the commercial drug. Most of the impurities detected in the recovered MTX were similar to those in the commercial drug with respect to chromatographic behavior and ultraviolet spectrum. Three new impurities detected in the recovered drug appeared to include the 7-hydroxy-metabolite of MTX. Uric acid was also present as a contaminant at a level of approximately* 1%. *Urine-derived MTX inhibited dihydrofolate reductase* in vitro *and was effective in tumor-bearing mice to a degree equivalent to commercial MTX. Two patients were treated with MTX isolated from urine resulting in favorable antitumor response and no adverse reaction.*

INTRODUCTION

MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-glutamate, is a folate antagonist that has been widely used in the chemotherapeutic treatment of a variety of neoplastic diseases [1]. More recently, high dose MTX followed by leucovorin rescue has been used successfully in combination with other antineoplastic agents in the treatment of osteogenic sarcoma [2–5]. Increasing interest in high dose MTX therapy will no doubt result in a markedly greater demand for this drug. High doses of 50–300 mg/kg could require as much as 25 g MTX per dose. The need for such large quantities of MTX presents an economic problem for many European countries hindering the use of high dose therapy. For this reason the present study was undertaken to determine the feasibility of the isolation of MTX from the urine of treated patients for re-use.

Pharmacokinetic studies of MTX administered in 6-hr infusions at high doses and followed by leucovorin rescue have been reported [3, 6, 7]. Following infusion MTX is rapidly cleared from the plasma with an initial half-life of 2–3 hr and is largely excreted in the urine. Although earlier studies on the metabolism of MTX in man at conventional low doses revealed only trace amounts of MTX metabolites [8, 9], a recent report by Jacobs *et al.* [10] has shown 7-hydroxy-MTX to be a significant urinary metabolite in patients receiving high doses. However, the level

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of the 7-hydroxy-metabolite was still found to be 10% or less of the excreted MTX.

The present study describes a relatively easy and efficient procedure for the recovery of MTX from the urine of patients after receiving high doses. Evidence is presented that recovered MTX is chemically similar to and has the same therapeutic effectiveness as the initially administered commercial drug.

MATERIALS AND METHODS

Chemicals

The MTX reference standard (Lot no. 55192) and pharmaceutical MTX in 1 g vials were both products of Lederle Laboratories, Pearl River, New York and were supplied by the Drug Development Branch, Drug Research and Development, National Cancer Institute, Bethesda, Maryland. Leucovorin (5-formyltetrahydrofolate; calcium salt) was purchased and N^{10}-methylpteroylglutamic acid was obtained as a gift from Lederle Laboratories. All other chemicals were of the highest commercial grade available.

Source of recovered MTX

Five patients, 3 males and 2 females, ranging in age from 18 to 42 yr, were treated for osteogenic sarcoma with a chemotherapy regimen alternating vincristine-high dose MTX, cyclophosphamide and adriamycin [5]. The treatment schedule was as follows: day 1, vincristine, 1.5 mg/m^2 i.v.; day 2, MTX, 200 mg/kg in a 4-hr infusion (preceded by the administration of sodium bicarbonate to maintain urinary pH between 7 and 8.5) followed by leucovorin rescue, 9 mg every 6 hr for 3 days; day 15, cyclophosphamide, 40–60 mg/kg i.v.; day 28, adriamycin, 1.5 mg/kg i.v.; day 42, cyclophosphamide 40–60 mg/kg i.v.; day 56, cycle repeated. No other drugs were given during the vincristine-MTX administration.

Isolation of MTX from urine

MTX was purified from urine collected for 24 hr from patients after the commencement of a 4-hr infusion of 10–18 g MTX (200 mg/kg). The urine was collected in glass containers and frozen until time of processing. The following steps were carried out under subdued light and at 4°C. The urine was first filtered through a Whatman GF/c glass fiber filter to remove particulate material. This was followed by ultrafiltration through a PM10 Diaflo membrane (Amicon Corporation, Lexington, Massachusetts) to remove high molecule weight material. The alkaline filtrate initial pH 8.0–8.3, was acidified with 3N HCl to pH 4 to precipitate MTX. The precipitate was collected by centrifugation at 2000 × *g* for 10 min and washed 3 times with 600 ml 10^{-3} N HCl. The MTX precipitate was redissolved in distilled water by the addition of 1 N NaOH. The MTX concentration was at least 5 mM at pH 8.0. After filtration through a glass fiber filter to remove undissolved material, calcium phosphate (2 g/mmole MTX) was added while stirring and maintaining the pH at 8.0. After stirring for 2 hr the calcium phosphate was removed by glass fiber filtration and the filtrate was again passed through an ultrafiltration membrane as before. The calcium phosphate treatment and second ultrafiltration step were included for the removal of pyrogens (personal communication; Organon Nederland B.V., Oss, The Netherlands). MTX was again precipitated by the addition of HCl to pH4, washed with 10^{-4} N HCl followed by absolute ethanol and finally dried with nitrogen. The recovered MTX was further dried under vacuum in the presence of NaOH pellets and stored refrigerated with dessicant.

Chromatography

MTX preparations were analyzed for impurities by ion-exchange chromatography on a DEAE cellulose (DE-52, Whatman Ltd., Kent, England) column 0.9 × 28 cm [11]. The column was eluted with a linear gradient of 0.1–0.4 M ammonium bicarbonate, pH 8.3, at a flow rate of 2.0 ml/min. Gradient elution was preceded by a 30-min elution period with the low concentration buffer. The eluate was either monitored at 254 nm using an LKB Uvicord and recorder or collected in 3.1 ml fractions and the absorbance measured at 260 and 370 nm.

Spectrophotometry

Ultraviolet absorption spectra were performed on a Perkin–Elmer 124 recording spectrophotometer.

MTX assay

MTX levels were determined by either (a) ultraviolet absorbance at 370 nm in 0.1 N NaOH based on the molar absorptivity of 7.0×10^3 or (b) the enzyme-inhibition assay [12]. In the latter method the source of dihydrofolate reductase was *Lactobacillus casei* (New England Enzyme Center, Boston, Massachusetts) and the MTX standard was of Lot No. 55192 from Lederle Laboratories.

Test for pyrogens

MTX preparations were tested in rabbits for the presence of pyrogens according to the procedure and standards described by the *U.S. Pharmacopoeia* XIX [13]. Solutions of MTX, 30 mg/ml, in 4.3% sodium bicarbonate were first treated with activated asbestos (Seitz Werke GmbH, Bad Kreuznach, W. Germany), 5 g/l, and filter-sterilized using a 0.22 μm filter before injected in test animals at a dose of 200 mg/kg. Asbestos treatment immediately before sterilization was routinely used for the removal of traces of pyrogens in the preparation of solutions suitable for injection. Drug preparations were regarded as "pyrogen-free" according to U.S.P. standards if the maximum individual temperature increase was no greater than 0.6°C and the sum of the temperature increase per 3 rabbits was no greater than 1.4°C.

Evaluation of in vivo *antineoplastic activity*

The comparative antitumor effectiveness of commercial MTX and of drug preparations recovered from urine was evaluated against a series of murine leukemias. $CD2F_1$ mice of both sexes (20–22 g, obtained from Charles River Italy, Calco, Italy) were transplanted i.p. with 10^5 cells of the compatible L1210, P388 or L5178Y leukemias, whereas BALB/c hosts were used for the syngeneic Moloney virus-induced LSTRA lymphoma. Tumor cell viability was routinely checked microscopically by Trypan-blue dye exclusion and only preparations with 95% viability accepted. Graded doses of the compounds were administered i.p. in saline and their effectiveness expressed by the T/C% value, i.e. the ratio, expressed as per cent, of the median survival time (MST) of the treated over control group. Experimental groups consisted of at least 10 mice, each experiment was repeated 3 times and a gross autopsy was performed on each animal to confirm tumor presence at death. Statistical analysis was performed by Duncan's Test.

RESULTS

Isolation of MTX from urine

Urine collected from patients during 24 hr after the initiation of high dose MTX therapy contained 75–90% of the administered dose. Purification of MTX from urine by acid precipitation, calcium phosphate treatment and ultrafiltration yielded over 60% recovery of the drug present (Table 1). Following the initial filtration steps to remove particulate material and high molecular weight substances, a sample of urine was fractionated by DEAE cellulose chromatography and the eluate monitored at 254 nm. Figure 1 shows the elution profile of MTX before and after acid precipitation from urine. MTX eluted between 2.4 and 3 hr as the dominant peak. Several minor peaks were detected especially in the early part of the elution before MTX. After the urine was acidified and the MTX precipitate washed and redissolved, chromatographic analysis demonstrated that 4–5 peaks eluting during the first hour were largely removed (Fig. 1B). Subsequent steps in the purification procedure, calcium phosphate treatment and repeated ultrafiltration and acid precipitation, failed to remove the remaining ultraviolet-absorbing impurities since the chromatographic profile of the final MTX preparation was essentially unchanged. (Fig. 4).

Purity of recovered MTX

Ultraviolet spectral analysis indicated that recovered MTX was similar to commercially available MTX, both having identical absorption maxima at 258, 300 and 370 nm (Fig. 2). Absorption maxima for the Lederle product purified by DEAE cellulose chromatography were recorded at 258, 301 and 371 nm. Table 2 presents the molar absorptivities at the observed absorption maxima calculated on the basis of a molecular weight of 454. According to previously reported values for molar absorptivities of 23.0×10^3, 22.4×10^3 and 7.0×10^3 at the maxima 257, 302 and 360 nm, respectively [1], a degree of purity of

Table 1. *Recovery of MTX from urine of patients after high dose MTX*

Purification step	Volume (ml)	MTX* (mM)	(g)	%Recovery
Crude urine after GF/c glass fiber filtration	2830	7.1	9.0	100
First ultrafiltration	2780	7.0	8.8	98
First acid precipitation followed by redissolving	1880	8.5	7.3	81
Calcium phosphate treatment	1930	7.1	6.2	69
Second ultrafiltration	1900	7.1	6.1	68
Second acid precipitation and final recovery of crystals	—	—	5.6	62

*MTX concentration was determined by the molar absorptivity at 370 nm of $7.0 \infty 10^3$

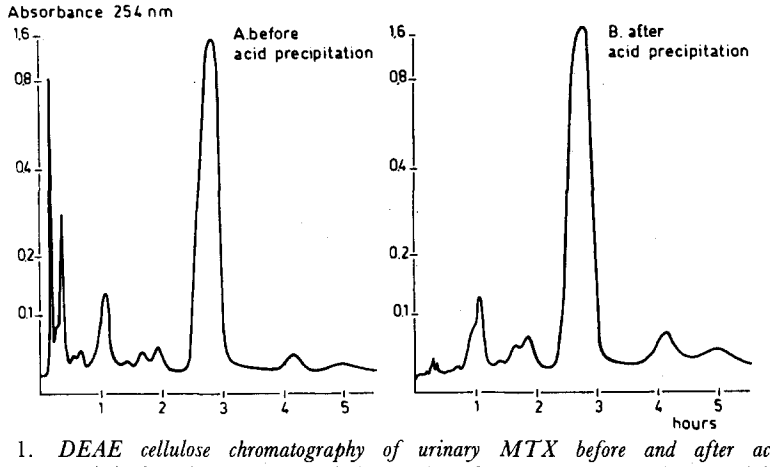

Fig. 1. *DEAE cellulose chromatography of urinary MTX before and after acid precipitation.* (*A*) *Sample chromatographed was* 1.4 ml *of a patient's urine containing* 7.1 mM *MTX and which had been filtered with a GF/c glass fiber filter followed by ultrafiltration.* (*B*) *Sample chromatographed was derived from the same urine as in A. MTX was first precipitated from the urine as described in the text and redissolved in distilled water with the aid of* NaOH. *The final MTX concentration was* 8.5 mM *and* 1.2 ml *were applied to the column. Chromatography was performed as described in the text.*

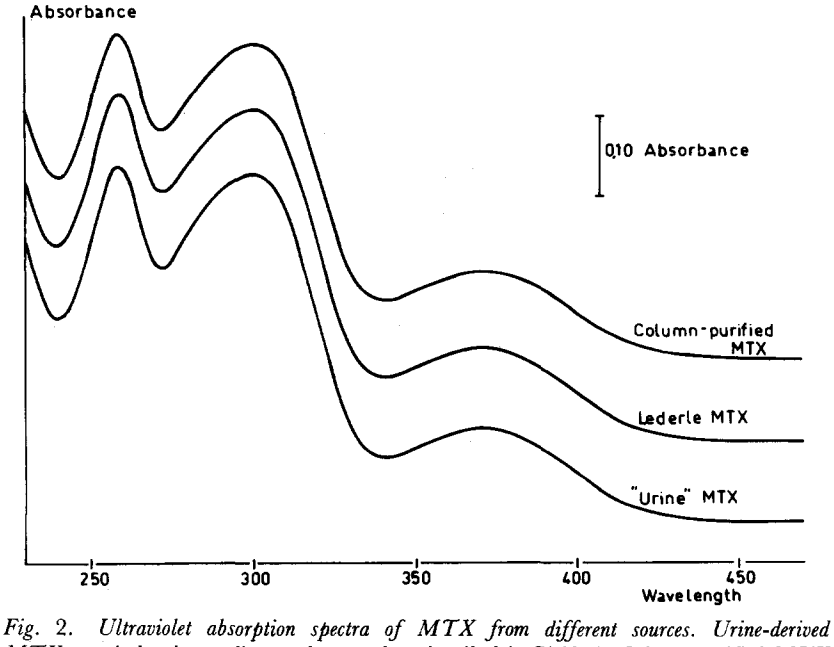

Fig. 2. *Ultraviolet absorption spectra of MTX from different sources. Urine-derived MTX was isolated according to the procedure described in Table* 1. *Column-purified MTX was obtained after fractionation of commercial MTX on DEAE cellulose (see Fig.* 3). *All solutions were prepared in* 0.1 N NaOH *at a concentration of* 2×10^{-5} M *MTX.*

recovered MTX was estimated to be 89–101% compared to 86–97% for the commercial drug.

The biochemical activity of recovered MTX was quantitatively determined according to its inhibition of dihydrofolate reductase. A 5.0 mg/ml solution of recovered MTX was found to be 11.0 mM or 100% as active as the reference standard.

Commercially available MTX preparations for clinical use contain several impurities arising from the synthetic process or degradation, including N^{10}-methylpteroylglutamic acid and 4-amino-4-deoxy-N^{10}-methylpteroic acid [9, 11, 14–20]. Figure 3 illustrates the chromatographic fractionation of the Lederle drug used in the present study as the initially administered drug, demonstrating the presence of 7 peaks in addition to the MTX peak (peak 5). Absorption at 260 and 370 nm by 5 of the 7 non-MTX peaks (and by 6 of the peaks in 0.1 N NaOH, Table 3) indicated that most of these impurities had spectral properties characteristic of pteridine compounds. Based on relative peak area, peak 5, representing MTX, accounted for 94% of the absorbance at 260 nm. Peaks 3, 4 and 6 were the most significant impurities at 1.0, 1.9 and 2.4%, respectively. The sum of the areas of peaks 1, 2, 7 and 8 was approximately 0.5% of the total.

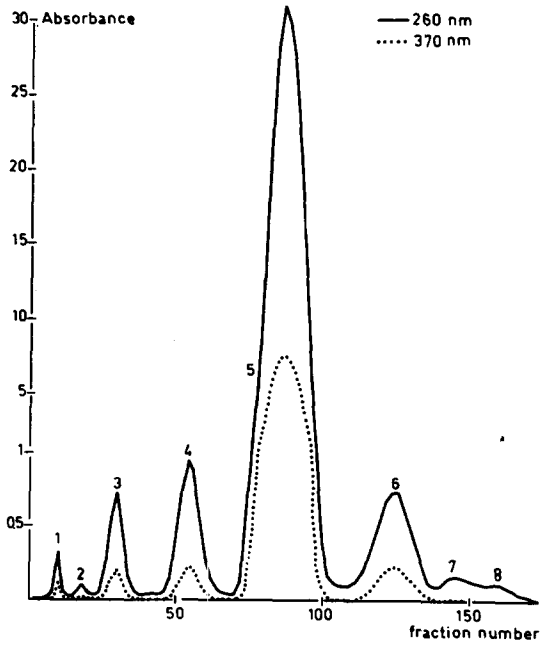

Fig. 3. DEAE cellulose chromatography of commercial MTX. 33 mg of Lederle MTX in 0.5 ml 0.1 M ammonium bicarbonate was chromatographed as noted in the text and 3.1 ml fractions were collected. Peaks were numbered according to order of elution.

Figure 4 illustrates the elution profile for a representative MTX sample purified from urine. The 7 impurities originally present in the commercial drug (Fig. 3) also appeared to be present in the recovered MTX preparation and were assigned the same numbers based on similarity in elution times. In addition, two new species were detected with different elution times and were assigned peak numbers 9 and 10. All impurity peaks demonstrated absorption at both 260 and 370 nm. Quantitation of peak areas at 260 nm indicated that the recovered drug was 93% MTX. Compared to the initial commercial product, peak 3 was increased (1.8%), peak 4 decreased (0.9%) and peak 6 decreased (1.6%). Peaks 1 and 2 were decreased but still detectable. Peaks 7 and 8 were increased, but could not be easily quantitated due to poor resolution. Regarding the new peaks observed,

Table 2. Absorption maxima (λ_{max}) and molar absorptivities (ε) of recovered and commercial MTX in 0.1 N NaOH

MTX	λ_{max} (nm)	$\varepsilon \times 10^{-3}$
Lederle	258	22.4
	300	21.4
	370	6.0
Urine-derived	258	23.0
	300	22.6
	370	6.2

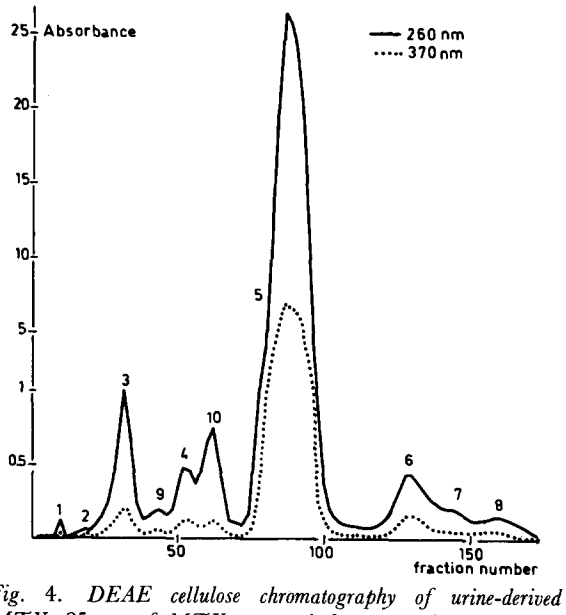

Fig. 4. DEAE cellulose chromatography of urine-derived MTX. 25 mg of MTX recovered from a patient's urine as described in Table 1 was chromatographed under the same conditions as in Fig. 3. Peaks were assigned numbers corresponding to those peaks in Fig. 3 with similar elution times. Peaks eluting in fractions 39–47 and 57–68 were not present in the elution profile of commercial MTX and were assigned numbers 9 and 10, respectively.

*Table 3. Absorption maxima of impurities of commercial and recovered MTX fractionated by DEAE cellulose chromatography**

Lederle MTX				Urine-derived MTX			
Peak No.	Absorption maxima (nm)			Peak No.	Absorption maxima (nm)		
1	254		365	1	258		368
2	251		355	2	255		357
3	258	302	370	3	258	295	366
4	259	284	370	4	258	285	369
5	258	301	371	5	258	300	370
6	255	301	368	6	254	301	365
7	259	282	370	7	258	281	369
8	258			8	259	302	
				9	261	290	370
				10	263	297	394

*Peak fractions were from the chromatographic separation in Figs. 3 and 4. Absorption spectra were determined after the addition of NaOH to a final concentration of 0.1 N.

peak 9 was just detectable, approximately 0.2%, and peak 10 was more notable at 1.4%. In other recovered MTX preparations from different patients, MTX as peak 5 represented 90–95% and peak 3 less than 4% of the total absorbance at 260 nm. Variation in the percentage of the other impurity peaks did not exceed ±0.5%, except for peak 10 which was in some samples less than 0.5%.

Peak fractions from the chromatographic fractionations of the initial commercial MTX and the recovered drug shown in Figs. 3 and 4 were characterized by ultraviolet spectrophotometry and their effect on dihydrofolate reductase activity. Table 3 lists the absorption maxima recorded between 230 and 450 nm. Chromatographic peaks 1, 4, 6 and 7 of recovered MTX corresponded to the respective peaks found in the commercial drug regarding both absorption maxima and the absorbance ratio at 260 and 300 nm (data not shown). Peak 2 of recovered MTX had similar absorption maxima but differed in the 260/300 ratio when compared to the corresponding peak of the commercial drug. The increased absorbance at 300 nm could have been due to overlapping by the larger peak 3. Peak 3 of recovered MTX differed largely from peak 3 of commercial MTX by a shift in absorption maxima from 302 to 295 nm and a decreased 260/300 ratio. Uric acid was found to co-elute with peak 3 providing an explanation for the spectral change due to the absorption maximum at 292 nm for uric acid. Furthermore, an assay of peak 3 for uric acid by the colorimetric phosphotungstic method indicated the presence of 0.2 mg uric acid per 25 mg of recovered MTX. A difference in spectral properties was found in peak 8 of recovered MTX compared to that of the initial commercial drug. There was an appearance of an absorption maximum at 302 nm which also resulted in a decreased 260/300 value. The absorption spectrum also demonstrated a shoulder at 340–350 nm and was similar to that of 7-hydroxy-MTX [20, 21]. Peaks 9 and 10, both unique for the recovered drug preparation, had spectral characteristics similar to MTX. However, peaks 9 and 10 differed from MTX having absorption maxima at 290 and 394, respectively.

Chromatographic peak fractions of both commercial and recovered MTX were examined for their effect on dihydrofolate reductase activity (Table 4). All fractions of either drug preparation demonstrated an inhibitory effect on enzyme activity which differed in degree. The assay system was routinely used to measure MTX levels in plasma at a maximum sensitivity of 10^{-9} M. Thus, the inhibitory effect on dihydrofolate reductase was expressed in MTX equivalents i.e. the equivalent concentration of MTX (reference standard used without further purification) required to produce the same degree of inhibition. Peak 5 exhibited 1600 and 1300 μM MTX equivalents for 33 mg of commercial drug and 25 mg of urine-derived drug, respectively. Peaks 3, 4 and 7 demonstrated 1.4–21 μM MTX equivalents or less than 2% relative to peak 5. Peak 3 was the most active in the inhibition of dihydrofolate reductase compared to the other impurities found in either drug preparation. Inhibition of enzyme activity by other impurity fractions was de-

*Table 4. Inhibition of dihydrofolate reductase by impurities of commercial and recovered MTX fractionated by DEAE cellulose chromatography**

Lederle MTX		Urine-derived MTX	
Peak No.	MTX equivalent† (μm)	Peak No.	MTX equivalent† (μm)
1	0.011	1	0.05
2	0.022	2	0.002
3	8.0	3	21
4	3.2	4	0.65
5	1600	5	1300
6	0.8	6	0.5
7	1.4	7	5.0
8	0.34	8	0.75
		9	0.5
		10	0.9

*Peak fractions were from the chromatographic separation in Figs. 3 and 4.

†Degree of inhibition of dihydrofolate reductase was expressed relative to the reference standard MTX (Lederle, Lot. No. 55192).

Table 5. Removal of pyrogens from recovered MTX

Purification procedure†	No. of rabbits	Temperature increase mean (range)
	Commercial MTX (Lederle)	
None	4	0.2 (0–0.3)
	Urine-derived MTX	
1. Acid precipitation	6	2.1 (1.0–3.4)
2. Calcium phosphate treatment, acid precipitation	6	1.2 (0.6–1.9)
3. Ultrafiltration acid, precipitation, calcium phosphate treatment, acid precipitation	4	1.2 (0.5–1.7)
4. Ultrafiltration, acid precipitation, calcium phosphate treatment, ultrafiltration, acid precipitation	7	0.3 (0–0.6)

*Maximum temperature in °C during 3 hr period after injection.

†All samples tested were prepared for injection as described in Materials and Methods. Samples prepared according to procedure 4 were ultrafiltered for a third time immediately prior to preparation for injection.

tectable but estimated at less than 0.1% with respect to peak 5.

Removal of pyrogens

Table 5 shows the results of the effect of different purification procedures on the removal of pyrogens. The commercial MTX preparation produced a mean maximum temperature increase of 0.2°C, the highest recorded at 0.3°C. MTX recovered from urine by only acid precipitation produced a marked pyrogenic reaction with a mean increase in temperature at 2.1°C despite treatment with asbestos prior to testing. Calcium phosphate treatment was effective in reducing the pyrogenic reaction lowering the temperature increase to 1.2°C. The use of both calcium phosphate treatment and ultrafiltration did not result in the further removal of pyrogens

as indicated by the observed temperature increase of 1.2°C. The use of larger quantities of calcium phosphate to improve pyrogen removal was not examined due to a significant reduction in yield of MTX from urine. However, additional ultrafiltration steps, three in all including one immediately before preparation for injection, yielded a MTX solution which produced a mean temperature increase of 0.3°C with a range of 0.6°C meeting the U.S.P. standards for a pyrogen-free parenteral solution.

In vivo *antineoplastic activity of recovered MTX*

The effectiveness of recovered MTX was compared to the commercial drug in the treatment of mice inoculated with various leukemias. Three MTX preparations derived from urine differing in extent of purification were examined. No statistically significant differences in antileukemic activity could be observed in our testing conditions between commercial MTX and urinary MTX whether the latter was isolated by only acid precipitation, recovered as described in Table 1, or even further purified by column chromatography. As shown by representative data in Table 6, single injections on day 1 of these compounds were moderately effective on P388, L5178Y and LSTRA leukemias, significant and equivalent increases in survival being obtained with doses of MTX and all urine-derived MTX preparations above 10 mg/kg i.p. Similar results were obtained when a more prolonged (day 1–15) treatment schedule was employed in L1210-bearing mice (Table 7). It may be seen that whereas doses of 10 mg/kg of the 4 compounds were uniformly toxic in this condition, the degree of prolongation in survival recorded after doses of 0.75–3 mg/kg were not statistically different for the commercial and urinary drug preparations.

Clinical use of recovered MTX

In two patients treated with 1 and 7.5 g of recovered MTX, respectively, the effectiveness of the urine-derived drug was clearly demonstrated, while no adverse side-effects were observed. Before administration, recovered MTX was prepared for injection and tested for the presence of pyrogens as described in Materials and Methods. Only drug preparations which passed U.S.P. standards and thus pyrogen-free were used in clinical studies. Nonetheless, body temperature was monitored continuously during the 48-hr period during and after MTX administration.

The first patient was a 63-yr old female with a poorly differentiated squamous cell carcinoma of the epiglottis. Because of infiltration of the tumor in the surrounding of the larynx, the tumor had been only partially resected. Postoperative treatment consisted of the administration of 1 g MTX every week. MTX was given in a 24-hr infusion and was followed by leucovorin rescue. The remainder of the tumor, which could be seen at laryngoscopy postoperatively, was no longer visible after 4 courses of MTX which included 3 treatments with recovered MTX. This apparently complete remission had been achieved without any clinical complications due to the urine-derived drug.

The second patient was a 78-yr old female who had been referred to the oncology unit with an inoperable squamous cell carcinoma of the tongue and had been treated previously with radiotherapy. She received a 24-hr infusion of 1 g MTX every 3 weeks followed by leucovorin. The tumor showed no change during 4 treatment cycles. It was decided to proceed with MTX in a regimen as recently described by Pitman and Frei [22]. MTX was administered weekly in gradually increasing doses from 1 to 7.5 g. Following successive doses of 1 g recovered MTX and 2.5 and 5 g doses of commercial MTX, no increase in tumor size was observed. After the 7.5 g dose using urine-derived MTX, a significant reduction in tumor size was noted. Although exact measurement of the tumor was difficult there was no doubt that a good partial remission had been achieved with high dose MTX recovered from urine. Again, no adverse reactions due to the urine-derived drug were observed.

DISCUSSION

The present report is an initial investigation into the feasibility of recovering MTX given in high doses from the urine of treated patients for the purpose of re-use. MTX was isolated from urine in high yield allowing the recovery of more than half of the initial dose administered. Isolation of MTX from urine by acid precipitation proved to be an effective method for obtaining a drug preparation of a purity similar to that of commercial MTX. Both inhibition of dihydrofolate reductase *in*

Table 6. Antineoplastic activity of single doses of commercial MTX and MTX recovered from urine on different murine leukemias

Compound	Dose (mg/kg/i.p.)	P388		LSTRA		L5178Y	
		MST*	T/C%	MST*	T/C%	MST*	T/C%
Controls	—	14.1	—	11.2	—	17.0	—
MTX, commercial	40	17.9	122	16.3	145	28.5	150
	30	15.3	108	16.1	143	23.0	134
	20	15.1	107	16.2	142	20.1	117
	10	15.0	106	13.5	120	19.6	114
	5	13.8	97	13.1	116	17.3	101
MTX, urine-derived†							
A	40	18.3	131	18.3	163	29.5	172
	30	18.0	127	16.5	147	23.3	136
	20	16.8	119	15.3	136	21.0	122
	10	16.1	114	17.3	154	20.1	117
	5	13.8	97	14.3	127	18.8	109
B	40	17.1	121	16.6	148	26.3	153
	30	16.8	119	16.3	145	23.5	137
	20	16.5	117	16.8	150	21.6	126
	10	14.0	99	16.5	147	19.3	113
	5	15.6	110	14.5	128	17.3	98
C	40	16.5	117	16.8	150	27.1	158
	30	13.6	96	17.7	158	23.4	138
	20	18.1	128	16.3	145	21.3	124
	10	16.8	119	17.5	156	21.0	122
	5	14.6	103	14.3	127	19.6	114

*Mean survival time in days. Tumour cells (10^5 for L5178Y; 10^4 for LSTRA; 10^6 for P388) were transplanted i.p. on day 0; drugs were administered on days 1 and five; CDF_1 mice were used for P388 and L5178Y leukemias and BALB/c mice for LSTRA lymphoma.

†MTX was purified from urine by either acid precipitation alone (A) or according to the procedure in Table 1 (B). C was the same as B, but further purified by column chromatography as shown in Fig. 4 (peak 5).

Table 7. Effect of repeated injections of commercial MTX and MTX recovered from urine on L1210 *leukemia**

Compound	Dose (mg/kg i.p.)	MST	T/C%
Controls	—	10.0	—
MTX, commercial	10	9.0	90
	3	19.5	195
	1.5	17.0	170
	0.75	14.0	140
MTX, urine-derived†			
A	10	6.5	65
	3	20.5	205
	1.5	18.5	180
	0.75	16.5	165
B	10	7.5	75
	3	19.0	190
	1.5	19.0	190
	0.75	17.0	170
C	10	7.0	70
	3	19.0	190
	1.5	18.0	180
	0.75	14.0	140

*10^5 cells were implanted i.p. on day 0 in $DC2F_1$ mice and drugs were administered from day 1 to 15.

†Purification of samples A, B and C are described in Table 6.

vitro and antitumor effectiveness in mice by urine-derived MTX was quantitatively equivalent to that observed with the commercial drug. Although recovered MTX and the commercial drug were essentially identical with respect to percent purity and biochemical activity, quantitative and qualitative differences were noted in the impurities detected by DEAE cellulose chromatography.

The estimation of the impurities in MTX preparations in this study was done by calculation of the relative peak areas of absorbance at 260 nm after chromatographic fractionation. Although such a determination of the level of impurities is limited to ultraviolet-absorbing substances and cannot yield absolute mass or molar quantities, it can be used to obtain relative values helpful in the comparison of different MTX samples. Chromatographic data indicated that commercial MTX represents 94% of the product based on absorbance at 260 nm in agreement with recently reported values [15, 17]. The urine-derived preparations were found to be 90–95% MTX. The major ultraviolet-absorbing impurity found in the commercial drug was identified as N^{10}-methylpteroyglutamic acid by its chromatographic and spectral properties when compared to a standard reference compound. The presence of this pteridine derivative was estimated to be 2.4%, consistent with the value 2.7% reported by Tong *et al.* [17]. These latter investigators also found in their analysis of commercial MTX by high pressure liquid chromatography one other notable impurity which was estimated to be 1.6% and identified as 4-amino-5-deoxy-N^{10}-methylpteroic acid. An impurity peak (peak 4, Fig. 3) was detected in the commercial drug in the present study which eluted before MTX and was found to represent 1.9% of the total absorbance at 260 nm. This impurity was not identified by comparison with the spectral and elution properties of a known standard compound, but was likely to be 4-amino-4-deoxy-N^{10}- methylpteroic acid based on previously reported chromatographic elution characteristics and the relative level detected.

These impurities mentioned above and several others having spectral properties characteristic of pteridines which were also present in the commercial drug were apparently present in recovered MTX at approximately equal or decreased levels. The unidentified impurity peak eluting before MTX was increased in the urine-derived drug. However, in the latter MTX preparation uric acid was detected in this chromatographic fraction. The amount of uric acid present was determined to be 0.2 mg per 25 mg of the MTX preparation. This was not surprising since uric acid is a major constituent of urine and is precipitated at acid pH. 7-HydroxyMTX was detected in the recovered drug and estimated to be less than 1% based on relative peak areas, indicating that the metabolic loss of MTX was low. No effect was observed by the presence of these low levels of 7-hydroxy-MTX and uric acid on the activity of recovered MTX *in vitro* or *in vivo*. Despite the known limited solubility of both of these compounds, no adverse effects were noted during the clinical use of recovered MTX at least in two patients. Any possible toxicity due to these impurities may have been circumvented by routine alkalinization (with sodium bicarbonate) prior to high dose MTX treatment. Regarding the two ultraviolet-absorbing contaminants (peaks 9 and 10, Fig. 4) found in urine-derived MTX but not detected in the commercial drug, they also exhibited spectral properties similar to MTX and produced inhibition of dihydrofolate reductase activity. It is conceivable that these impurities are metabolites of MTX or of the pre-existing impurities in the commercial drug preparation.

Patients undergoing MTX chemotherapy often receive simultaneously other drugs which could appear in the MTX preparations isolated from the urine. The patients whose urine was used in this study for the isolation of MTX received no antiemetics since little nausea due to high dose MTX treatment was experienced. Although vincristine was given 24 hr before high dose MTX in these patients, a recent study has shown that in man only about 9 and 12% of the vincristine dose is excreted in the urine during the first 24 and 72 hr, respectively, after administration, the drug being excreted primarily in the feces [23]. Since leucovorin (5-formyltetrahydrofolate) was given shortly after the start of high dose MTX, its presence in the form of its known metabolites [24], including 5-methyltetrahydrofolate in recovered MTX was possible. However, only 45 mg of leucovorin was given during the 24-hr period after the MTX infusion, about 1% or less of the amount of MTX which could be recovered. No leucovorin could be detected in the urine-derived drug and no decrease in pharmacologic (antitumor effectiveness) activity was observed with the recovered MTX compared with the commercial drug. It should be also noted that the removal of impurities from urine-derived

MTX by ion-exchange chromatography produced no significant difference in antitumor activity when tested in mice.

Removal of pyrogens from MTX preparations recovered from urine was necessary before testing in patients. This was accomplished with calcium phosphate treatment and ultrafiltration. MTX isolated from urine by only acid precipitation produced a severe pyrogenic reaction in rabbits despite treatment of the test solution with asbestos prior to testing. Asbestos, although now restricted in the United States, is still used in Europe for the removal of pyrogenic substances by absorption for the preparation of parenteral solutions. Asbestos treatment alone appeared to be ineffective in the removal of pyrogens. Attempts to eliminate pyrogens by calcium phosphate treatment, also based on adsorption, proved to be helpful but not completely satisfactory. The addition of ultrafiltration steps to the purification procedure did result in a drug preparation that was pyrogen-free according to U.S.P. standards [13]. Since pyrogens, which are primarily lipopolysaccharides released from bacterial cell membranes, are of a macromolecular nature [25], ultrafiltration would seem to be best suited for their removal from solutions of MTX. Ultrafiltration membranes used in this study were capable of retaining 10,000 mol. wt species, although not quantitatively. The use of other, more efficient molecular filtration membranes (e.g. Pellicon membranes, Millipore Corporation, Bedford, Massachusetts) especially suitable for pyrogen removal in pharmaceutical processing would undoubtedly be an improvement and possibly would eliminate the necessity for calcium phosphate treatment and multiple ultrafiltration steps.

The present study demonstrates the feasibility of the recovery of MTX from patients on high dose MTX therapy for re-use. The isolation procedure is relatively simple and the product is comparable to the commercial drug based on chromatographic analysis, *in vitro* inhibition of dihydrofolate reductase and antitumor effectiveness in mice. The urine-derived MTX preparation can be made pyrogen-free and suitable for use in patients. The trial of high dose treatment with recovered MTX in two patients has demonstrated desired antitumor activity without adverse effects. Further studies are needed, however, to fully identify all impurities present in both the commercial and recovered drug and to establish their possible toxicity or involvement in the antitumor action of high dose MTX therapy. Such information would provide a better understanding of the potential problems that could arise from the use of MTX recovered from urine. Improvements on the removal from the recovered drug of the residual impurities, especially those not present in the initial commercial drug would be just as desirable. The practical recovery of gram quantities of MTX with minimal levels of impurities from the urine of treated patients has been demonstrated; however it has not been determined at this time whether the recovery of urine-derived MTX is possible without significant accumulation of impurities. Indeed, the continuous re-utilization of MTX would increase its availability and would further reduce restrictions on high doses due to the cost.

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The Effects of Cyclophosphamide Treatment on the Epithelium and Stroma of the Urinary Bladder

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Abstract—*In a series of* 28 *patients treated with cyclophosphamide for non-urological diseases who developed haemorrhagic cystitis during treatment, seven showed "tumour-positive" cells in the urine sediment. The interval between the institution of cyclophosphamide treatment and the first urological symptom varied between* 4 days *and* 10 yr. *Cytoscopical examination revealed abnormalities of the bladder lining due to stromal changes, which explained the clinical picture. Two of the patients developed papillary tumours (histological grade* III).

In 4 *of the* 7 *patients the cytological abnormalities disappeared after the cessation of the therapy; in the other* 3 *they persisted fatally. In the one case in which autopsy was permitted, the results showed transitional cell carcinoma of the bladder with metastases in the lungs and bone marrow.*

INTRODUCTION

CYCLOPHOSPHAMIDE (Endoxan®) has been widely used since 1957 in the treatment of cancer and, more recently, as an immunosuppressive drug given before transplantations and in numerous non-neoplastic diseases such as rheumatoid arthritis and systemic lupus erythematosus. It is administered in an inactive form and is metabolized, mainly in the liver, into a potent cytotoxic agent [1, 2]. The drug and its metabolites are eliminated via the urine [3]. Since the bladder, unlike the renal pelvis and the ureters, is in prolonged close contact with urine, the urogenital toxic effects of the drug are seen predominantly in this organ [4–6].

Like the chemically related nitrogen mustard, cyclophosphamide is mutagenic [3, 7, 8] and carcinogenic [9]. Atypical cells are found in the urine of cyclophosphamide-treated patients [10, 11] and infiltrative bladder cancer has been reported in association with this drug [12–16].

In addition to the epithelial changes, cyclophosphamide may also induce alterations in the subepithelial vessels and stroma of the bladder leading to sterile haemorrhagic cystitis [16–18].

This retrospective study concerned a series of 28 patients treated with cyclophosphamide during the last 4 yr, all of whom had been free of urinary-tract diseases when this therapy was initiated, During treatment, 7 of these patients developed urological complaints and cytological abnormalities in the urinary sediments and the drug was withdrawn.

In two of these cases bladder cancer was diagnosed. Further cytological and histological investigations were undertaken to evaluate the character of these stromal and epithelial changes.

MATERIALS AND METHODS

Seven patients (Table 1) who developed urological symptoms with an abnormal cytological picture during cyclophosphamide treatment were analyzed.

The clinical histories of these 7 patients were reviewed with special attention to urological symptoms prior to cyclophosphamide treatment. Correlations were sought between the cystoscopical and histological findings (biopsy or autopsy) and the clinical data. Furthermore, the daily and total doses of cyclophosphamide and the interval between the beginning of the treatment and the onset of urological symptoms were determined. All of the patients had received the cyclophosphamide orally for a period of five days, every three or four weeks except one (No. 7), who had been given the drug intravenously. In cases 1, 2 and 6 the drug was administered in

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Table 1. Clinical, histological and cytological data of 7 patients treated with cyclophosphamide. Follow-up data after cessation of therapy

No.	Age in yr	Sex	Reason for CP treatment	Total dose	Daily dose	Time lapse	Urinary symptoms	Cystoscopy	Cytology	Histology	Time	Urological symptoms	Cytology	Histology
1	36	m	lymphocytic lymphoma	50 g	300–400 mg,	45 months	hematuria frequency	edematous red areas	positive		18 months	transient	negative	none
2	64	m	reticulum cell sarcoma	32 g	350 gr	13 months	burning hematuria frequency penile pain	edematous, red	positive	denuded edematous stroma, vascular changes	6 months	transient	positive	patient died (no autopsy) clinically of reticulum cell sarcoma
3	84	m	squamous cell carcinoma of lung	162 g	350 mg.	10 yr	hematuria	papillary tumors	positive	grade 3* carcinoma	12 months†	transient	negative	none
4	75	m	Kahler's disease	8.05 g	75 mg.	25 months	frequency hematuria voiding pain	papillary tumors	positive	grade 3* carcinoma	6 months	persistent	positive	patient died of widespread bladder carcinoma
5	52	f	Kahler's disease	27 g	200–300 mg.	4 yr	hematuria, burning	edematous, diffuse red	positive		24 months	transient	negative	none
6	46	f	mamma carcinoma	6 g	300 mg.	4 months	hematuria, scalding	red	positive		2 months	persistent	positive	patient died (no autopsy) clinically of mamma carcinoma
7	13	f	preparation for bone marrow transplant	11 g	2850 mg.	4 days	frequency, severe hematuria	edematous, red, teleangiectasia papillary folds	positive		3 months	transient	negative	none

*Graded according to the Bergkvist grading system [6].

†In this case the cytological report remained positive over a period of six months following the transurethral resection of the tumor, and the bladder mucosa was reported by the cystoscopist as papillomatous. After 12 months the cystoscopic findings and the cytology were negative, thus the epithelium returned to the normal state.

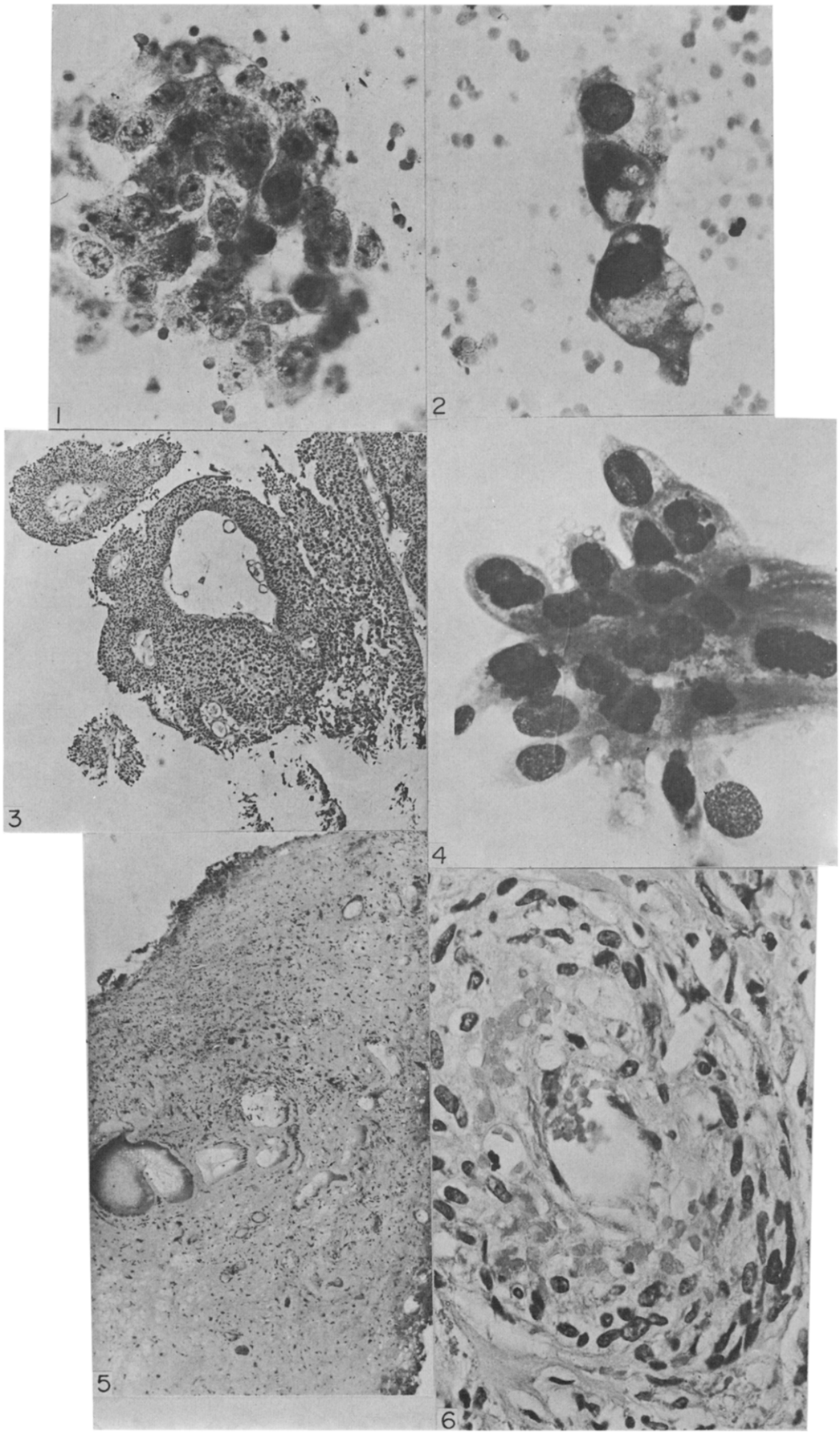

Fig. 1. *Papanicolaou-stained morphologically malignant cells. Case no.* 3: *histological confirmation of cancer.* × 500.

Fig. 2. *M.G.G.-stained highly abnormal urothelial cells with changes mimicking radiomimetric effect.* × 500.

Fig. 3. *Tissue section of grade* 3 *papillary tumor, case No.* 3. × 125.

Fig. 4. *M.G.G.-stained highly abnormal cells with chromatin changes, not visible in this black and white microphotograph, case No.* 6. × 500.

Fig. 5. *Bladder biopsy of case No.* 2. *Note edema and teleangiectasias. The epithelium is absent* ("*denuding cystitis*"). *The stroma does not contain a heavy inflammatory infiltrate. The dark dots in the stroma represent multinucleate fibroblast.* × 500.

Fig. 6. *Thickening of the intima of a small vessel in the bladder stroma, case No.* 2. × 500.

combination with vincristine and prednisone, in case 4 with prednisone only, and in cases 3, 4 and 7 without any other drug.

RESULTS

The patients had been free of urological disease before treatment and had no history of urological anomalies. The relevant data (age and sex, reason for cyclophosphamide treatment, daily and total doses given, interval between the beginning of therapy and onset of symptoms and the cytological and, if available, the histological findings) are given in Table 1. The daily dose varied between 75 and 2850 mg and the total dose between 6 and 162 g. In some cases the period before the onset of symptoms was short (4 days in No. 7); in others it was much longer (10 yr in No. 3).

In all cases the urological complaints had started with signs of bladder irritation (e.g. frequent urination, nocturia, burning and scalding) followed by haematuria. The urine was sterile in all cases. Treatment to control the bleeding was required in only one case (No. 7). During the follow-up period, which varied from 2 to 24 months, the urological symptoms proved to be transient in five cases and persistent in the other two (Table 1). The duration of the urological complaints did not always correspond with that of the cytological abnormalities.

Cystoscopically, the bladder lining showed a picture closely resembling that of carcinoma *in situ* [19], including edema, redness and teleangiectasia.

The cytological investigation performed at the onset symptoms revealed a large number of morphologically malignant cells in 4 cases (Fig. 1) and only a few "tumour-positive" cells in the other 3 cases (Nos. 1, 5 and 7). In the latter group the cytology returned to normal after termination of the therapy. Of the other four patients, two developed bladder cancer (Nos. 3 and 4) and 2 died of the primary malignancy (Nos. 2 and 6); an autopsy could not be performed in these two cases. In the cases with gross tumour growth, many papillary cancer cell groups were found. In one case the cell changes (Fig. 2) resembled those described by Forni *et al.* [11] as due to irradiation. The histological diagnosis of the urothelial tumours in patients 3 and 4 was grade 3 carcinoma (Bergkvist grading system [20]) (Fig. 3). Cytologically, there were no differences between the histologically confirmed and non-confirmed cases (Figs. 1 and 4). Stromal and vascular changes were seen in the biopsy specimens of the patients with urothelial neoplasms as well as in patient 2, in whom no macroscopical tumour growth was detected. The stromal changes included edema, fibroblastic proliferation, and degeneration (Fig. 5); the vascular changes consisted of proliferation and thickening of the intima of some vessels, fibrin deposits in the vessel wall, and dilatation of the vascular wall with ectasia (Figs. 5 and 6).

Except for the haematuria, the clinical picture in the patients with cyclophosphamide-induced haemorrhagic cystitis closely resembled that seen in our cases of primary carcinoma *in situ* [19].

DISCUSSION

Haemorrhagic cystitis induced by cyclophosphamide therapy is rare in patients in whom a high fluid intake is maintained and retention of urine in the bladder is relatively short [21]. The reported frequency of this side-effect ranges between 2 and 40% (8% in a recent article by Jayalakshmamma [22]), possibly as the result of differences in dosage. The combination of haemorrhagic cystitis and urothelial cell abnormalities may be even more rare [11].

The development of papillary tumours, as in two of our patients, very probably represents the end of a long process starting with epithelial regeneration (characterized by atypia and hyperplasia of the newly formed epithelium) and the formation of papillary folds [23–25]. These changes have even been found several months after cessation of the therapy [11]. "Tumour-positive" cells were found in the absence of clinical cancer in patients given cyclophosphamide for a relatively short time (Nos. 2 and 6), whereas the combination of true tumour cells and clinical cancer was seen in patients treated for a longer period (Nos. 3 and 4). It will take many years to determine the long-term significance of the atypical epithelium observed in those of our patients who have not yet developed cancer since cytological changes can precede clinical urothelial cancer by as much as 20 yr [26].

It is noteworthy that all of our patients showed an abnormal cystoscopical picture. The characteristic redness of the mucosa is a result of the increased vascularity and teleangiectasia. The dilated vessels bleed easily, as sometimes occurs even after withdrawal of the drug (patients 2 and 6).

In the acute phase, necrosis may be seen in the lamina propria of the bladder and in the blood vessels [5, 23]. Late effects of cyclophosphamide therapy include stromal fibrosis, as observed in children by Johnson and Meadows [27].

Although cytological changes caused by cyclophosphamide were first described many years ago [11], reports of fully developed bladder cancer have only been published in the last few years, the first by Worth in 1971 [16]. It seems likely that the carcinogenic potential of cyclophosphamide was less evident when this form of treatment was less successful than it is now. At present, however, the drug is widely used for non-neoplastic diseases, which means that a large number of patients are being exposed to the risk of developing this iatrogenic bladder cancer.

Since cytological changes are also found in patients without urinary bladder complaints [10, 11], it seems advisable to perform a cytological investigation of the urine of all patients treated with cyclophosphamide. Such investigations would make it possible to identify those patients who run the risk of developing bladder cancer and would also provide invaluable data on the incidence and nature of these cyclophosphamide-induced bladder changes.

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The Effect of Cyclophosphamide upon Mitoses in the Cornea of Rats, a Circadian Dependent Effect

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Abstract—*In these studies, the corneal epithelium was used to determine:* (1) *The magnitude and duration of mitotic inhibitory effect of cyclophosphamide upon a rapidly proliferating tissue of the host.* (2) *Whether isoproterenol-induced circadian phase-shifts, obtained through alterations of cell cycle kinetics, could in any way minimize the mitotic inhibitory effects (toxicity) of subsequently administered cyclophosphamide. The data presented suggest:* 1. *That marked differences in the duration of the mitotic inhibitory effect can result from the administration of cyclophosphamide at different "times of the day".* 2. *That pretreatment with isoproterenol enhances the circadian dependent responses of the rat's cornea to cyclophosphamide.* 3. *That with administration of cyclophosphamide at or shortly after the beginning of the animal's daily phase of full wakefulness, beneficial responses may be expected to reach maximum circadian levels.*

INTRODUCTION

STUDIES over the past few years have indicated that the magnitude of toxic as well as beneficial responses to drugs depend significantly on the time of day (or circadian phase) of their administration [1]. Chronotherapy, or the administration of drugs on schedules adjusted to circadian rhythms of the host should, therefore, permit the use of higher drug dosages, possibly at more frequent intervals. Evidence supporting this concept and extending it to cancer chemotherapy has been presented [2–11].

Badran *et al.* [9] observed a greater suppression of tumor growth in animals receiving cyclophosphamide at a time of the day in which circadian peak levels (as determined in non-treated control tumors) of cell division were present in the transplanted mammary tumor tissue. They suggested that the circadian dependent growth inhibitory effect of this anticancer agent was directly related to the cell division rates present in the tumor tissue at the time of drug administration [9]. More recently, with the use of BDF_1 mice bearing L1210 leukemia, Cardoso *et al.* [5] have shown that the toxic as well as the therapeutic responses to cyclophosphamide (180, 240, 300 and 360 mg/kg) are indeed circadian dependent. From those dose–response studies, the calculated LD_{35}'s (maximum death rates of 38% at 1800 hr) were found to be 360 mg/kg and 254 mg/kg, respectively for the best (1800–2100 hr) and worse (0600–0900 hr) circadian-phases treatment groups [10].

To verify the validity of Badran's proposition and to gain further knowledge of the mechanisms responsible for the circadian dependent responses to cyclophosphamide, additional studies were carried out to determine: (1) The magnitude and duration of mitotic inhibitory effect of cyclophosphamide upon a rapidly proliferating tissue of the host, (2) Whether isoproterenol-induced circadian phase-shifts [4, 6, 11] obtained through alterations of cell cycle kinetics [6, 12] could in any way minimize the mitotic inhibitory effects (toxicity) of subsequently administered cyclophosphamide.

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MATERIALS AND METHODS

Male Sprague–Dawley rats with weight ranging from 100 to 120 g at arrival from Harlam Industries, Indiana were housed (six/cage) in a windowless room with temperature and humidity maintained, respectively, at 23°C and 50%. Artificial lights were kept on from 0600 to 1800 hr. The animals were kept in this environment for at least one week prior to their use in the experiments. Purina lab chow and water were available *ad lib*. Single doses of either cyclophosphamide (300 mg/kg) (Mead Johnson Research Center, courtesy of Dr. K. W. Wheeler) or isoproterenol HCl (25 mg/kg) (Sterling-Winthrop Research Institute, courtesy of Dr. F. C. Nahod) were administered to the appropriate groups of rats, by the intraperitoneal route at the times indicated under "Results". The rats were sacrificed by decapitation at the times indicated under "Results". The corneas were collected, fixed and stained as previously described [2]. Mitoses were counted with the use of a microscope (occular 10×, objective 100×) with a micrometer grid disc inserted in the occular piece. One hundred grid fields were counted in each cornea. All phases of mitoses were included with exception of those cells in early prophase and late telophase.

RESULTS

Magnitude and duration of cyclophosphamide's mitotic inhibitory effect

In Fig. 1 we are presenting the results obtained in two separate experiments. In the first (Panel A), a single dose of cyclophosphamide, 300 mg/kg, was administered at 0900 hr (arrow in the figure) by the i.p. route to the appropriate group of rats. Two hours after treatment, cyclophosphamide-treated- and non-treated control rats were sacrificed. Subsequent time-groups of control and treated rats were sacrificed at the times indicated in the figure. The results obtained with the administration of the agent at 1800 hr (2nd experiment) are presented in Panel B. All data obtained are expressed as mean number of mitoses/100 fields ± S.E. (5 rats/mean) of the mean values. Comparisons (*t*-test) between control and corresponding cyclophosphamide-treated groups of rats sacrificed between 1100 hr on day 1 and 0600 hr on day 3 (45-hr time span, Panel A), revealed the following *P* values: <0.005, <0.005, <0.025, <0.05, <0.02, <0.001, <0.001, <0.001, <0.05, <0.05 and >0.05. Similarly, for those rats treated at 1800 hr (24 hr time-span, Panel B) and sacrificed between 2200 hours on day 1 and 1800 hr on day 2, the *P* values were: <0.005, <0.001, <0.005, <0.02, <0.001, <0.05 and >0.05. Thus, a profound inhibition of mitoses without time alterations of the circadian rhythm, resulted from drug administration at either 0900 or at 1800 hr of the day. On the other hand, while this inhibition of cell division extended over 39 hr among the animals treated at 0900, it extended only over 21 hr among rats which received the drug at 1800 hr of the day.

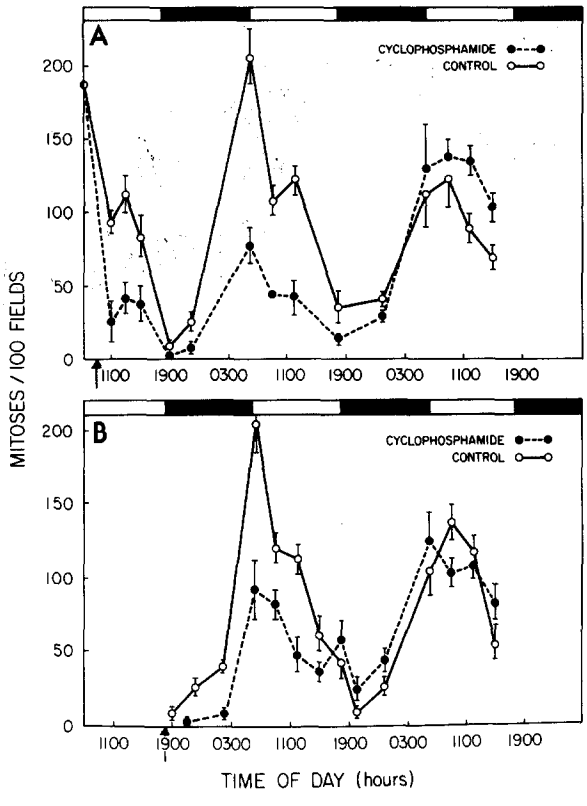

Fig. 1. The effect of cyclophosphamide upon mitoses in the cornea of rats. A single 300 mg/kg *dose of cyclophosphamide was administered (arrow) to rats at either* 0900 *(Panel A) or at* 1800 hr *of the day. Subsequently, treated* (5) *and non-treated control* (5) *rats were sacrificed at the times indicated in the figures. The mean and S.E. of the mean values (mitoses/100 fields) are presented. The full and open horizontal bars shown on the top of Panels A and B indicate the hours of darkness* 0600 hr) *and light* (0600–1800 hr) *to which the animals were exposed throughout the experiment.*

Isoproterenol-induced phase-shifts displacement in time of circadian peaks and troughs of mitotic activity

In Fig. 2, results obtained with the administration of a single (25 mg/kg) dose of isoproterenol at either 0600 or at 1500 hr of the day, are presented in Panels

A and B, respectively. Inspection of the curves reveal outstanding differences in the circadian mitotic rhythm in rats treated with the agent at 0600 hr, when compared with controls; as shown in Panel A, its effect extended over 3 successive circadian mitotic cycles. *First cycle:* displacement of the circadian mitotic peak from early morning (0600 hr) to mid-afternoon (1500 hr). *Second cycle:* pronounced suppression of the mitotic

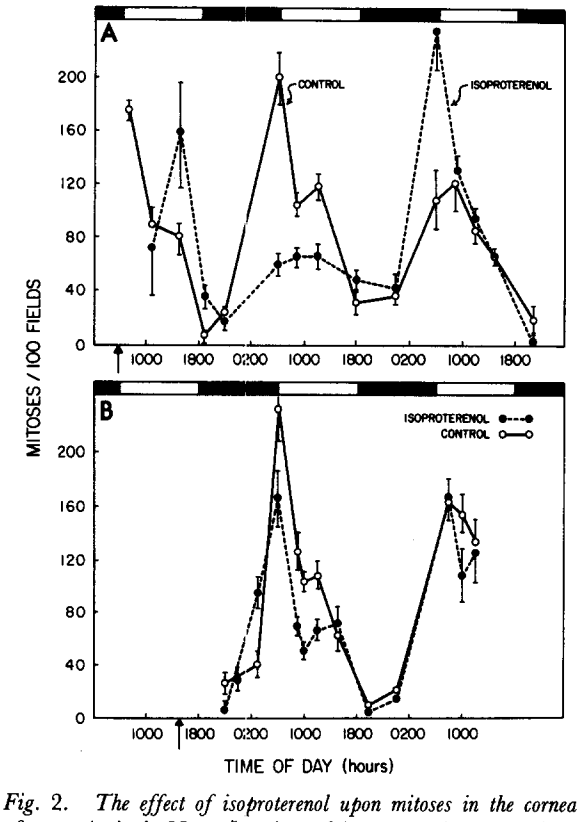

Fig. 2. The effect of isoproterenol upon mitoses in the cornea of rats. A single 25 mg/kg *dose of isoproterenol was administered (arrows) to rats at either* 0600 *(Panel A) or at* 1500 hr *(Panel B). Subsequently, control and treated rats were sacrificed at the times indicated in the figures. The mean* (5 *rats/time group) and S.E. of the mean values (mitoses/*100 *fields) are presented. The full and open horizontal bars represent hours of darkness and light, as in Fig.* 1.

cycle. The P values obtained from comparisons (t-test) between isoproterenol suppressed groups and corresponding controls were as follows: at 0600 hr $P<0.001$; at 0900 hr $P<0.005$ and at 1200 hr $P<0.001$. *Third cycle:* "Rebound" mitotic activity, with peak levels of cell division significantly above controls ($P<0.005$), occurring at the appropriate circadian phase, i.e. transition from dark to light, at 0600 hr of the day. Discrete suppression of mitoses without changes in the overall rhythm characterized the responses to isoproterenol administered at 1500 hr (Panel B)

The effects of cyclophosphamide upon mitoses in the corneas of rats pretreated with isoproterenol

In Fig. 3, the results obtained with the administration of cyclophosphamide (300 mg/kg) to rats previously treated with isoproterenol (25 mg/kg), are presented. In Panel A, the treatment times were 0600 and 0900 hr, respectively for isoproterenol and for cyclophosphamide. Both in Panels A and B, the results obtained with drug combination

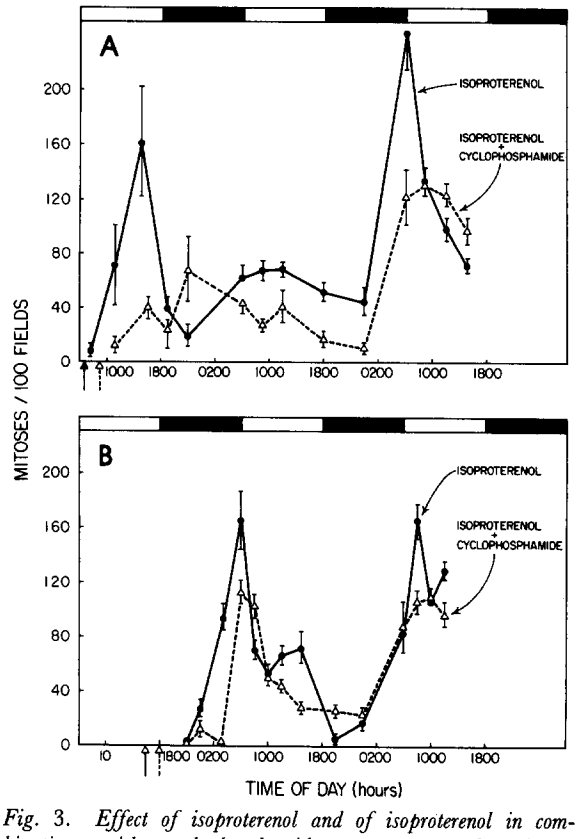

Fig. 3. Effect of isoproterenol and of isoproterenol in combination with cyclophosphamide, upon corneal mitoses. Isoproterenol and cyclophosphamide were administered (25 mg/kg *and* 300 mg/kg *respectively), singly or in combination, to rats at the times indicated by the arrows in the figures. The results obtained with the drug combination are compared with those obtained with the administration of isoproterenol alone. The mean* (5 *rats/time group) and standard error of mean values (mitoses/*100 *fields) are presented. The full and open horizontal bars represent hours of darkness and light as maintained in the animal quarters.*

are compared with the responses observed after the administration of isoproterenol alone. Figure 3 shows: (1) the profoundly different responses which follow drug administration (singly or in combination) at different circadian phases (Panel A vs Panel B); (2) that pre-treatment with isoproterenol enhances (compare with Fig. 1), the circadian dependent effects of cyclophosphamide: (3) that relatively low levels of cell division were present in the corneas of isoproterenol-treated

rats at the time of cyclophosphamide administration: (4) the absence of mitotic "rebounds" in the corneas of rats treated with the combination isoproterenol-cyclophosphamide.

DISCUSSION

The therapeutic index of many drugs depends, significantly, on the time of day (circadian dependent response) of their administration [1]. This knowledge has stimulated interest in chronotherapy as an approach to increase the efficacy of cancer chemotherapy. Improvements have been obtained [2–11]. Nevertheless, drug induced toxicity still remains as a major limitation to cancer chemotherapy.

More recently, efforts have been directed toward a reduction of the degree of anticancer drug toxicity, demonstrated to be circadian dependent. To this end and in preparations for chronotherapy, manipulation of the number of normal and neoplastic cells in a given phase to the cell cycle was attempted using a non-cytotoxic agent such as isoproterenol. To date such results, although non conclusive, have been encouraging [6].

The present work expands on the above work, with the following objectives: (A) To gain understanding of the cause and/or mechanism(s) of circadian dependent effect of cyclophosphamide. (B) Verifying whether isoproterenol, a non-cytotoxic agent with known effects upon circadian mitotic rhythms [6, 11] and kinetics of the cell cycle [12] could, in any way, modify and/or minimize the cytotoxic effects of cyclophosphamide.

A. *Cause and/or mechanism(s) of the circadian dependent effect of cyclophosphamide*

Circadian phases of maximum and minimum mitotic activity, respectively at 0900 and 1800 hr of the day, were selected for the administration of cyclophosphamide [5]. As presented in Fig. 1, a profound inhibition of corneal mitoses resulted from drug administration at either circadian phase. On the other hand, the duration of the drug's mitotic inhibitory effect was found to extend over a considerably longer period of time in those animals treated at 0900 hr (39 hr) than in animals treated at 1800 hr (21 hr).

The significance of the above finding, in terms of cause and/or mechanisms of host toxicity, can be appreciated only in light of: (1) the established synchrony of circadian mitotic rhythms in fast proliferating tissues of the host [11]; (2) the demonstrated value of the rats corneal epithelium as a predictive system for the cytotoxic effects of anticancer agents upon fast proliferating tissues of the host [2, 7, 11]. Thus viewed, our findings suggest that a relationship of cause and effect may exist between duration of mitotic inhibitory effect and circadian dependent responses to cyclophosphamide.

B. *Pretreatment with isoproterenol in attempts to minimize the cytotoxic effects of cyclophosphamide*

For this purpose, the circadian dependent effects of cyclophosphamide upon the corneal epithelium were further investigated with the use of (a) isoproterenol induced "circadian phase shifts" (time displacements of circadian peaks and troughs of mitotic activity); (b) cyclophosphamide administration to control and to phase shifted animals. Initially, isoproterenol was administered alone, at different circadian phases, in order to identify the most desirable time for the subsequent administration of cyclophosphamide. As shown in Fig. 2, markedly different responses resulted from isoproterenol administration at either 0600 or 1500 hr. On the other hand, an immediate and pronounced fall in the rates of cell division was a common response to treatment at either hour. For the purposes stated above (a, b), the initial phases of suppression of cell division by isoproterenol were chosen for use in the subsequent isoproterenol-cyclophosphamide combination studies. The data in Fig. 3 demonstrate that in response to treatment with the combination isoproterenol-cyclophosphamide, profound changes of rhythm and rates of cell division do occur: *1st Type* (Panel A) an intense suppression of mitoses along with a concomitantly abolished circadian mitotic rhythm; *2nd Type* (Panel B) less intense inhibition of mitoses along with a preserved circadian mitotic rhythm. Thus, the results indicate that isoproterenol can influence the target tissue's response to cyclophosphamide and that, therapeutically speaking, either beneficial (treatment at 1500–1800 hr) or adverse (treatment at 0600–0900 hr) responses may be obtained from the administration of cyclophosphamide in combination with isoproterenol. The results also indicate that the overall magnitude of cyclophosphamide's mitotic inhibitory effect is not directly related to cell division rates present in the target tissue at the time of its administration: as shown in Panels A and B, low rates of cell civision were present in the

target tissue at the time of cyclophosphamide administration to either group.

Experiments have been planned to investigate further the effects of the above drug combination, as well as that of isoproterenol with cell cycle phase specific agents, in tumor bearing animals. From the foregoing, isoproterenol (administered at the "proper time") may prove capable of exerting a favorable influence upon the therapeutic index of cyclophosphamide and. theoretically, even more so on that of agents such as cytosine arabinoside.

In conclusion, the data presented suggest:

1. That marked differences in the duration of the mitotic inhibitory effect can result from the administration of cyclophosphamide at different "times of the day".
2. That circadian levels of mitoses may not, *per se*, be responsible for the differences in toxicity which follow cyclophosphamide administration at different circadian phases. On the other hand, the ratio cycling/non-cycling cells may be related to and explain the circadian dependency of the agent's effects.
3. That the effect of isoproterenol on the corneal epithelium cells is reversible, while that of cyclophosphamide is (on many cells) irreversible.
4. That pretreatment with isoproterenol enhances the circadian dependent responses of the rat's cornea to cyclophosphamide.
5. That with administration of cyclophosphamide at or shortly after the beginning of the animal's daily phase of full wakefulness, beneficial responses may be expected to reach maximum circadian levels.

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Quantitative Immunocytochemical Study of Plasma Cells in Skin Tumoral Stromal Reaction*

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Abstract—*Immunoglobulin producing cells in the perilesional infiltrate of various benign and malignant skin tumors were sought using peroxidase labelled anti-immunoglobulin antisera.*

The prominence of such cells in the infiltrate was in general proportionate to the malignancy of the lesion, malignant melanomata showing the fewest.

INTRODUCTION

THE INFLAMMATORY infiltrate around tumors probably develops as a host defense mechanism. This "stromal reaction" [1] is composed partly of immunologically competent cells. Little is known of the precise role of anti-tumoral immune reaction [2–12].

Immunocytochemical identification of immunoglobulin producing cells (IPC) in lymphoid formations of the digestive tract has been carried out using peroxidase conjugated anti-human immunoglobulin antisera [13, 14]. Similar techniques [15] were applied to the inflammatory infiltrates of various cutaneous hyperplastic and tumorous processes.

MATERIALS AND METHODS

Patients

One hundred and four cutaneous tumors from both sexes (age range 15–83 yr) were studied (see Table 1).

Table 1. Patients

Basal cell carcinomas	25
(2 cystic + 23 solid)	
Squamous cell carcinomas	16
Bowen's disease	4
Seborrhoeic warts	10
(basal cell epitheliomas)	
Keratoacanthomas	12
Benign intradermal naevocellular naevi	15
Malignant melanomas	22
7 superficial spreading melanomas	(SSM)
3 lentigo maligna melanomas	(LMM)
7 nodular melanomas	(NM)
5 recurrent SSM	

Preparation of tissues

Biopsies were embedded in paraffin after fixation in Bouin's solution for 24 hr. The maximum interval between embedding and labelling was 6 months.

Conjugates

Monospecific (anti IgA, anti IgG, anti IgM) peroxidase conjugated sheep anti-human immunoglobulin (Ig) sera were used (I. Pasteur, Paris). The sheep antisera were made specific for heavy chain determinants by solid-phase immuno-absorption. Specific antibodies were isolated on immunoglobulin-immunoabsorbents [16].

Immunoglobulins were conjugated to peroxidase according to the method of Avrameas and Ternynck [16]. Preparations contained 1.25 mg antibodies/ml. The optimal dilution determined by a previous titration was 1/20 for anti IgG and IgM sera and 1/30 for anti IgA sera.

Immunocytochemical technique

After dewaxing, 5 μm sections were washed in phosphate buffered saline (PBS) 0.01 M, pH 7.2 for 20 min at ambient temperature and subsequently incubated with the conjugates for 60 min at 37°C. The sections were then washed twice in PBS

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(5 and 25 min respectively) and in 0.2 M Tris–HCl, pH 7.6 for 10 min. The peroxidase activity was then revealed by Graham-Karnovsky media (5 mg 3.3′ diaminobenzidine (Sigma) in 10 ml 0.2 M Tris–HCl, pH 7.6 with 0.01 H202) for 8 min at ambient temperature, in darkness. The sections were again washed twice in PBS (5 and 25 min respectively) and counter stained with methyl green solution (0.25% Fast green FCF (Gurr) in distilled water + 1% acetic acid) for 10 min at ambient temperature. The preparation were rinsed for 10 min in distilled water, dehydrated and mounted in Canada Balsam.

Specificity

Specificity was determined using a sheep anti-rabbit Ig conjugate (I. Pasteur, Paris) on sections from the same tissue.

Table 2. Basal cell carcinomas (18 cases): Classes of IPC present in infiltrate and their staining intensity

	Number of tumors		
Presence of IPC	IgA PC	IgG PC	IgM PC
++(*)	8	10	4
+(†)	8	7	8
Total (out of 18)	16	17	12
Staining intensity			
Strong	12	7	5
Weak	4	10	7
Absence of IPC	2	1	6

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

Endogenous peroxidase activity was demonstrated by direct development with the Graham–Karnovsky media, without exposure to conjugate.

Histology

Sections were compared with haematoxylin–eosin–safranine stained specimens.

RESULTS

IPC were identified as large (12–15 μm) oval cells with eccentric nuclei with a clock-face chromatin pattern and abundant dark brown staining cytoplasm. IPC were found in cellular infiltrates, around or within the tumor (Figs. 1 and 2).

Cells with endogenous peroxidase activity (polymorphonuclear leukocytes, (PMN), histiocytes) could be identified by their morphology. Free melanin appeared as light brown clusters. Within melanophages, the mainly granular pigment stained lighter brown in colour than Ig. Connective tissue appeared deep green, with a slightly brownish tinge.

Basal cell carcinomas

None had either sebaceous, sweat gland or pilar differentiation [17]. Eighteen tumors showed IPC, in most of which all classes of Ig were observed (Table 2), although in 14 cases a predominance of IgG IPC was present (Table 3). In 7 cases, all with scanty infiltrate, no IPC was seen.

Squamous cell carcinomas

All three classes of Ig were represented among the IPC in nearly all 16 cases (Table 4) with a predominance of IgG (Table 3). All 16 lesions, 8 of which were ulcerated, were grade I (Broder's index) [18].

Table 3. Predominant immunoglobulin class in the IPC of the infiltrates of various types of tumors

		Number of tumors			
			Predominant Ig class		
Type of tumor		With IPC	IgA PC	IgG PC	IgM PC
Bowen's disease		2	0	2	0
Keratoacanthomas		6	0	5	1
Seborrhoeic warts		5	0	5	0
Naevo-cellular naevi (15 cases)		0	0	0	0
Basal cell carcinomas		18	4	14	0
Squamous cell carcinomas		16	1	15	0
Melanomas	LMM	3	2	1	0
	SSM	5	3	2	0
	NM	2	0	2	0

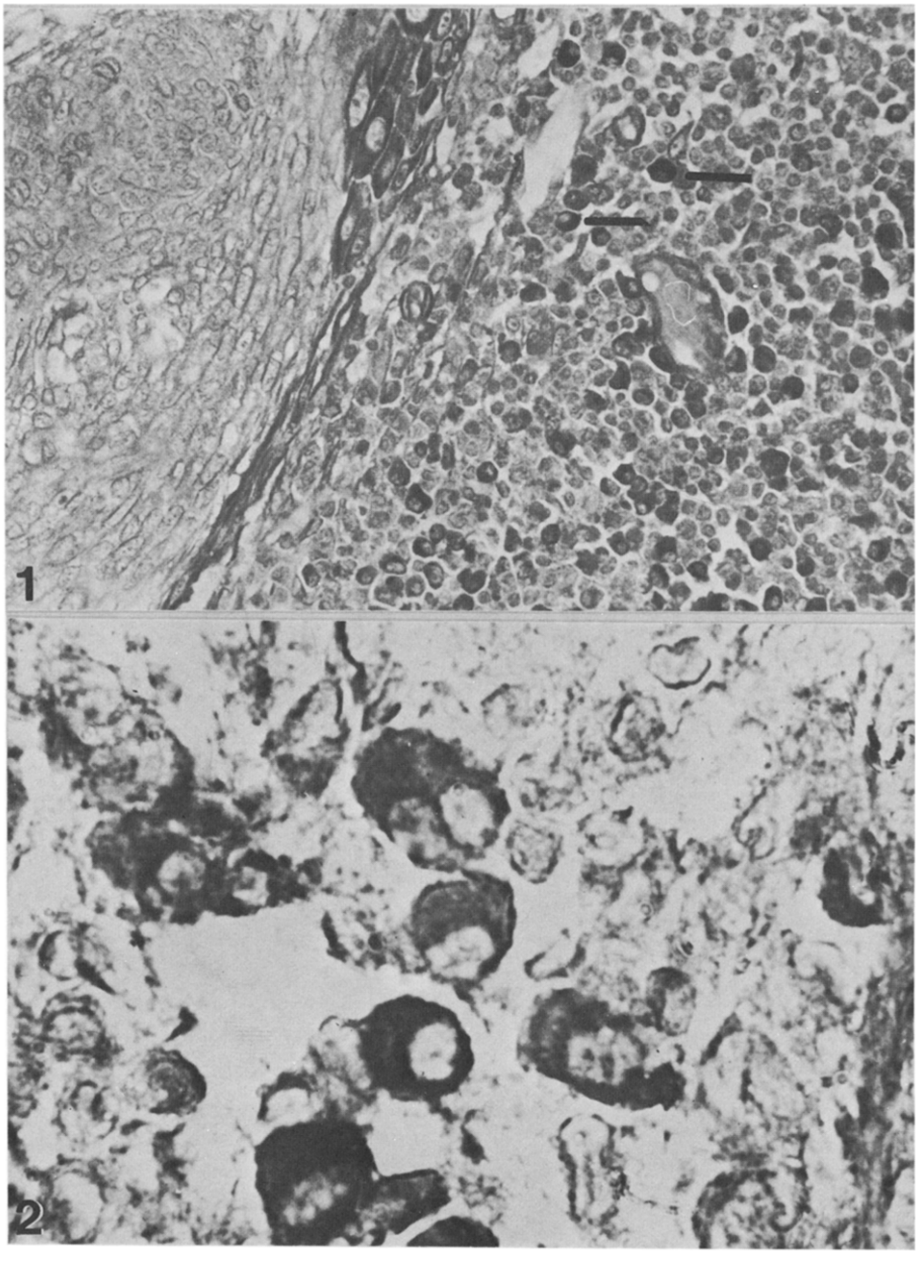

Fig. 1. *Basal cell carcinoma. Inflammatory infiltrate showing plasma cells* (*arrows*). *Immunolabelling with anti IgG serum conjugated with peroxidase* (× 180).

Fig. 2. *Plasma cells in basal cell carcinoma inflammatory infiltrate* (*detail*). *Immunolabelling with anti IgG serum conjugated with peroxidase* (× 1500).

Table 4. Squamous cell carcinomas (16 cases): classes of IPC present in infiltrate and their staining intensity

	Number of tumors		
Presence of IPC	IgA PC	IgG PC	IgM PC
++(*)	8	11	7
+(†)	6	5	6
Total (out of 16)	14	16	13
Staining intensity			
Strong	11	8	6
Weak	3	8	7
Absence of IPC	2	0	3

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

Table 5. Bowen's disease (2 cases): classes of IPC present in infiltrate and their staining intensity

	Number of tumors		
Presence of IPC	IgA PC	IgG PC	IgM PC
++(*)	0	1	0
+(†)	2	1	1
Total (out of 2)	2	2	1
Staining intensity			
Strong	2	1	0
Weak	0	1	1
Absence of IPC	0	0	1

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

Table 6. Keratoacanthomas (6 cases): classes of IPC present in infiltrate and their staining intensity

	Number of tumors		
Presence of IPC	IgA PC	IgG PC	IgM PC
++(*)	1	1	2
+(†)	4	5	4
Total (out of 6)	5	6	6
Staining intensity			
Strong	5	1	0
Weak	0	5	6
Absence of IPC	1	0	0

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

Bowen's disease (*Table* 5)

Two tumors were devoid of IPC. Where present, all Ig classes were seen.

Keratoacanthomas (*Table* 6)

IPC were seen in 6 out of 12 tumors, with a predominance for IgG IPC (Table 3).

Seborrhoeic warts (*basal cell papillomas*) (*Table* 7)

In 5 cases with little infiltrate, no IPC were seen. IgG IPC were predominant (Table 3) in the remainder.

Intradermal naevi

No IPC were seen.

Malignant melanomas (*Table* 7)

(a) Out of 7 SMM, 2 showed no IPC (scanty infiltrates). In 5 SSM with IPC, an intense peritumoral infiltrate (lymphocytes, plasma cells, melanophages, mononuclear cells, PMN) was observed. IPC were only found in upper dermis. Intensity of staining was weak. In one case, IPC comprised more than 1% of the infiltrate (Table 8). IgM IPC were seen only in one case. In 3 cases IgA IPC and in 2 cases IgG IPC were predominant (Table 3).

(b) In the 3 LMM, IPC were found in the upper dermis. IgA, with strong staining, predominated (Tables 3 and 8).

(c) IPC were seen in 2 NM only (Table 8), where they occurred lateral in the margins of the tumor in the superficial dermis only. The 5 NM with no IPC were devoid of infiltrate, or had few infiltrating cells.

(d) No recurrent melanomas had IPC.

Table 7. Seborrhoeic warts (5 cases): classes of IPC present in infiltrate and their staining intensity

	Number of tumors		
Presence of IPC	IgA PC	IgG PC	IgM PC
++(*)	1	2	1
+ (†)	3	3	3
Total (out of 5)	4	5	4
Staining intensity			
Strong	4	3	2
Weak	0	2	2
Absence of IPC	1	0	1

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

Table 8. Malignant melanomas: classes of IPC present in infiltrate and their staining intensity

Type of tumor	Presence of IPC	Number of tumors IgA PC	IgG PC	IgM PC
SSM (5 cases)	++(*)	0	1	0
	+(†)	4	1	1
	Total (out of 5)	4	2	1
	Staining intensity			
	Strong	1	1	0
	Weak	3	1	1
	Absence of IPC	1	3	4
LMM (3 cases)	Presence of IPC			
	++(*)	1	1	1
	+(†)	2	0	0
	Total (out of 3)	3	1	1
	Staining intensity			
	Strong	2	1	0
	Weak	1	0	1
	Absence of IPC	0	2	2
NM (2 cases)	Presence of IPC			
	++(*)	1	2	1
	+(†)	1	0	0
	Total (out of 2)	2	2	1
	Staining intensity			
	Strong	2	2	0
	Weak	0	0	1
	Absence of IPC	0	0	1

(*) More than 1% of IPC for the relevant Ig class.
(†) Less than 1% of IPC for the relevant Ig class.
The percentages being calculated in relation to all cells present in the infiltrate.

DISCUSSION

IPC are generally considered to be mature plasma cells [13–15]; younger lymphoplasmocytic cells may also possess cytoplasmic Ig. This might explain observed differences in morphology and staining intensity of IPC. For IgA, greater intensity might result from a higher intracellular concentration. Ultrastructural study of the infiltrates [15] showed the peroxidase staining to be situated in the ergastoplasm of plasma cells, presumably during Ig synthesis. Plasma cells and IPC may be considered to be identical.

Plasma cells cannot be recognized by enzyme-histochemical studies alone [2]; immunofluorescence methods [19] require immediate interpretation and do not permit correlation of IPC with other cells of the infiltrate or tumor. Immunocytochemical techniques on fixed tissues avoid these difficulties.

IgM IPC were found least frequently (Table 3). Predominance of IgG over IgM IPC occurs during secondary humoral immune responses [20, 21]. An inflammatory reaction to tumor growth may correspond to such an immune response, in view of its long-term evolution (IgA IPC are the predominant type in LMM and SSM only).

Presence of IPC varies according to the type of tumor:

IPC are absent or scanty in naevocellular

naevi and Bowen's disease, where the infiltrate is poorly developed. In seborrhoeic warts and keratoacanthomas IPC were absent in half of cases and few when present. There was no correlation between the presence of IPC and general histological pattern of the lesions.

Basal and squamous cell carcinomas exhibit the greatest number of IPC. No relationship was found between number and kind of IPC and ulceration, or stage of dermal invasion. All squamous cell carcinomas were grade I. This would support the hypothesis that the presence of IPC is related to the degree of keratinisation and differentiation [6].

In malignant melanomas, in contrast to other studies [22], presence of IPC does not appear to depend on ulceration in our cases. However, IgM IPC were seen only in ulcerated tumors, which may indicate participation of new immuno-competent cells in an amplified anti-tumoral reaction.

No IPC were observed in recurrent melanomas, in this and other studies [2]. Observation of numerous plasma cells in metastases [7] has been made in the case of only two primary melanomas of undefined type. Proportionately lower number of DNA synthetizing lymphoid cells have been seen in metastases [10, 11].

In SSM, LMM and 2 NM, IPC were only seen in the upper dermis, as in previous studies [2]. No relationship could be established between presence of IPC and various other parameters [22, 23] (cell type, mitotic index, vascular emboli, angiogenesis ...). In the prognosis of malignant melanoma, the depth of invasion and histogenetic type of tumor are considered to be important [22, 23]. IPC appear to be always absent at deeper levels of the infiltrate. In NM, where the prognosis is very poor, IPC were found superficially in only 2 out of 7 cases. In SSM and LMM, IPC were found in 80% of cases. Presence of IPC may reflect the degree of malignancy.

Thus 3 kinds of tumors may be distinguished:

(A) benign dysplastic or degenerative lesions, with rare IPC, and a possible predominance of IgM IPC, when seen, in this group only. The rarity of IPC may however be related to the paucity of infiltrate.

(B) tumors with weak or mild degree of malignancy (basal and squamous cell carcinomas) with dense stromal reactions, many IPC (IgG). Identification of lymphoid cells in these infiltrates has shown a predominance of T. lymphocytes (high T/B ratio >4) [4, 24]. In such tumors, both kinds of lymphoid cells of immune response are present.

(C) highly malignant tumors (SSM, LMM and NM), with rare IPC (IgG and IgA). T lymphocytes are significantly less numerous [3, 24]. Both T and B lymphoid cells are rare in such infiltrates.

This study does not define whether or not these immunoglobulins have a specific antibody function [21]. Peritumoral immunoglobulin elution assays are at present being carried out. These studies, like those concerning activated macrophages [25, 26], antibody dependent lymphocytotoxicity [27] and direct killer effect of activated lymphocytes [28, 29], may eventually define the various antitumoral immunological mechanisms.

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Cell Cycle Agents and Cyclophosphamide in the Treatment of Diffuse Histiocytic Lymphoma

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Abstract—*A regimen consisting of two three-day courses of large doses of methotrexate with leukovorin rescue, followed by cytosine arabinoside infusion, given during* days 1 *and* 7, *and on* day 15, *vincristine, cyclophosphamide and prednisone with a time interval of* 18–24 hr *between vincristine and cyclophosphamide was tested. A total of* 6 *such cycles were used in* 9 *patients with histiocytic lymphoma who had not received prior chemotherapy. All nine responded, with a complete remission in* 8. *Prolonged disease-free survival of* 48 *or more months were seen in* 3 *of the complete responders.*

The efficacy of the cell-cycle agents, methotrexate and cytosine arabinoside was demonstrated since they were used as the initial phase of therapy, during which time a 78% *response rate was observed.*

INTRODUCTION

In the past, the treatment of histiocytic lymphoma (HL), was associated with a high response rate but remissions were of short duration [1–3]. More recent reports indicate a marked improvement in duration of complete responses [4–6].

Levitt *et al.* [7] introduced the use of high doses of methotrexate (MTX) with leucovorin (LCV) rescue, followed by cytosine arabinoside (Ara-C) infusion, after initial induction with cyclophosphamide (CTX) and vincristine (VCR) in previously untreated patients with disseminated histiocytic lymphoma [7]. All patients had partial or complete remission (PR, CR) with a median survival of over 15 months. However, it was not clearly demonstrated that the favorable results were related to the addition of the MTX and Ara-C, since vincristine and high-dose cyclophosphamide were used for initial therapy. Previously, antimetabolites had been reported to have only limited antitumor effect in lymphomas [8, 9], and conflicting reports suggesting either synergism or antagonism [10, 11] with combination of Ara-C and MTX led to uncertainty regarding the effectiveness of these two agents.

As a preliminary phase, the present study explored the utilization of the antimetabolites alone in a group of patients with disseminated lymphoma resistant to a variety of routines of combination chemotherapy. Following demonstration of antitumor activity in half of these patients, the study was extended to a second phase utilizing MTX-Ara-C, followed by VCR, CTX and prednisone (PRD), in 9 patients with previously untreated histiocytic lymphoma.

MATERIALS AND METHODS

(A) In the preliminary phase of the study, MTX with LCV rescue followed by Ara-C were given to a group of 8 patients. No CTX, VCR or PRD was given. The drugs were given in the same dosage as in the second phase listed below, and the routine was repeated each week.

(B) The regimen of the second phase was as follows (Fig. 1): Day 1: MTX 30 mg/m^2 by mouth, every 6 hr times 4 was given, followed immediately by 150 mg/m^2 of Ara-C given intravenously in continuous infusion for 6 hr. Rescue was started with leucovorin 25 mg/m^2 every 6 hr times 4 intramuscularly 24 hr after the first

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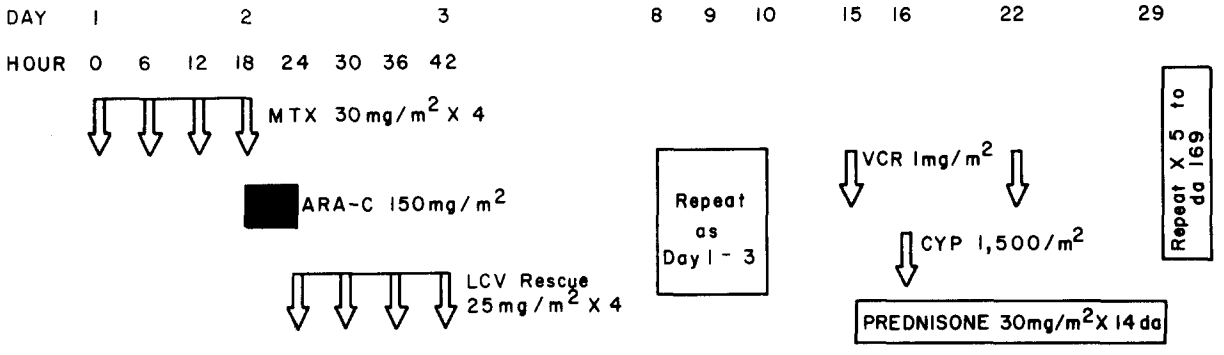

Fig. 1. Schema of therapy. Six such cycles were given over a 6-month period.

dose of MTX. Day 8: The treatment given on day 1 and 3 was repeated. Day 15: VCR, 1 mg/m^2 intravenously was given by push dosage and PRD 30 mg/m^2/days for 14 days, orally was started. Day 16: 18–24 hr after the VCR dose of day 15, a dose of CTX, 1500 mg/m^2 was given intravenously by push dosage. Day 22: VCR was repeated at the same dose as day 15. The first cycle was completed on day 28. Day 29: The second cycle was started.

Six such cycles constituted a course of therapy, following which no maintenance therapy was given.

All patients were admitted to the hospital for 24–36 hr for the Ara-C infusion and leucovorin rescue. The following dosage modification was utilized for bone marrow toxicity:

If granulocytes were more than 1000/mm^3 and platelets were more than 85,000/mm^3 on day of treatment, full doses of MTX and Ara-C were given. For counts below these levels, these drugs were omitted. CTX was modified to 75% when pretherapy platelet counts were from 65,000–85,000; and 50% for platelets of 45,000–64,000/mm^3. CTX was given at full dosage for pretherapy granulocyte counts of more than 1500, 50% for 1000–1500 and none for less than 1000/mm^3.

The VCR dose was reduced to 50% after appearance of significant weakness from neurotoxicity and omitted for over 50% reduction of expected muscle strength.

Therapy was discontinued if no response was seen after one full 28-day cycle of therapy, or if relapse occurred during therapy.

Patient selection

Preliminary phase. Eight patients with uncontrolled disseminated lymphoma who had failed to multiple combination chemotherapy programs, 6 with diffuse HL and 2 with mixed cellularity Hodgkin's Disease (HD) were treated in the preliminary phase. Most patients had WBC below 4000 and/or platelets below 100,000 from disease or former therapy.

Second phase. Nine patients with histologically confirmed disseminated diffuse histiocytic lymphoma, who had not had prior chemotherapy were admitted to this study. Three of them had received previous radiation therapy limited to 1–2 regions and 2 total nodal radiation, with complete response of tumor within radiation ports. All had normal WBC and platelet counts. Four had been recently diagnosed and had received no previous therapy. Five were male, 4 female. The range of age was 26–82 yr, with a median of 70 yr.

Complete remission was defined as complete disappearance of all measurable tumors.

Partial remission was defined as 50% or greater reduction in cross-sectional area of all measurable tumors.

No response was defined as progressive disease, no change, or minor improvement that did not qualify as a partial response.

Blood counts, biochemistry profiles, bone marrow aspirate and biopsy and radiographic studies of chest and abdomen were obtained in all patients before treatment. Blood counts were repeated before each dose of myelosuppressive drugs. Liver biopsy, lymphogram and radioisotope scans were obtained when indicated. Baseline studies included tumor measurements in two dimensions (except with bone marrow, spleen and liver involvement). Restaging including repeat bone marrow and liver biopsies when initially abnormal were repeated to confirm the existence of CR.

RESULTS

Preliminary phase

Four of the 8 patients treated during

Table 1. *Patient characteristics and response to therapy*

Patient	Age and sex	Predominant sites	Prior therapy	Response	Fate	Duration on study
1	60 F	Nodes, marrow	TNR*	CR	Died in remission, BM drug toxicity	3 months
2	68 M	Liver	RT,† mantle	CR	Died in remission, lung and liver fibrosis, drug toxicity	4 months
3	72 F	Nodes, marrow, soft tissue	None	CR	Died in remission with chronic lung disease and heart failure	5 months
4	82 M	Nodes, spleen	None	PR	Died in relapse	7 months
5	70 M	Nodes, spleen, SVC syndrome	None	CR	Died in relapse	9 months
6	58 M	Nodes, soft tissue	RT, mantle	CR	Died in remission with myocardial infarction	12½ months
7	26 F	Nodes, soft tissue	RT, shoulder	CR	Alive in CR	48+ months
8	73 F	Nodes, spleen	TNR	CR	Alive in CR	49+ months
9	77 M	Liver, nodes, soft tissue	None	CR	Alive in CR	53+ months

*TNR—Total nodal radiation.
†RT—Radiotherapy.

the preliminary phase showed partial response with the antimetabolites alone. None achieved CR. Responders included one with HD and 3 with HL. The duration of remission ranged from 6 to 92 days, with a median of 47 days. Two had fatal drug related hematologic toxicity (both were responders). Three others (two responders) had moderate hematologic toxicity, while three had little or no toxicity.

Second phase

I. *Response.* All 9 patients showed response, with CR in 8 (Table 1). Two patients of this group relapsed. One relapsed after CR and died at 9 months. The other relapsed from PR and died of disease at 7 months. Four died in remission, 2 with drug toxicity and two of unrelated disease. Three of those with CR had less than the programmed therapy (2 delinquent and 1 with severe prolonged infection), completing only 4, 10 and 14 weeks of therapy respectively and these three were still in remission at 48, 49 and 53 months after beginning therapy.

II. *Response rate to initial antimetabolite phase.* Seven of the 9 patients showed an antitumor effect sufficient to qualify for a PR in the first two weeks of the regimen during the MTX-Ara-C phase. None achieved CR during this initial period.

III. *Toxicity.* Six patients died during the study. Two deaths were directly related to drug toxicity; bone marrow aplasia and fulminant sepsis after the VRC–CTX–PRD phase in one and massive lung and liver fibrosis presumed to have been due to MTX toxicity after autopsy in the other (both were in CR). Two others died with lymphoma, in relapse from CR and PR. Two died of causes unrelated to the therapy or disease: one with severe congestive heart failure from underlying heart disease and chronic lung disease, and the other with acute myocardial infarction (both in CR).

It is important to emphasize that all but one were elderly patients with a median age of 70 yr and most had prior myocardial and vascular disease. Six of these 9 patients would not have qualified for the cooperative group protocols into which these patients might have been entered at this institution.

Aside from the one fatal reaction, hematologic toxicity was acceptable. During the first two weeks of each course of treatment, the MTX-Ara-C phase of therapy was followed by moderate thrombocytopenia but little if any leukopenia, while after the VCR–CTX–PRD phase, the reverse effect of leukopenia but much less thrombocytopenia was seen. Seven episodes of leukopenia of less than 2000/mm^3 occurred, requiring cessation of therapy for 2–3 weeks.

All these episodes occurred after the VCR–CTX–PRD phase of the study. Only two instances of thrombocytopenia of less than 100,000 occurred after the methotrexate-Ara-C phase.

All patients had severe alopecia. Nausea and vomiting occurred in all patients during Ara-C infusion and in some after CTX injection. No stomatitis was seen from the short periods of therapy with MTX. Only two patients manifested neurotoxicity of

sufficient severity to cause diminution of dosage of vincristine.

DISCUSSION

This study confirms the favorable results obtained by Levitt *et al.* [7] with introduction of antimetabolites for intensive treatment of histiocytic lymphoma. However, the therapeutic routine used in this study varied in three aspects from that of the Yale group.

First and most important was the introduction of MTX-Ara-C as the first modality of therapy. The efficacy of these two agents, with a 78% response rate, was clearly shown in the present study. However, this study does not demonstrate a preference in sequence of the agents, since none received VCR–CTX–PRD as first phase. The response rate reported here is approximately equivalent to that obtained by Levitt *et al.* utilizing CTX–VCR as first agents.

Second, the present regimen utilized careful sequential timing between the vincristine and cyclophosphamide. Experimental animal studies and clinical trials showed that the utilization of a time interval between antimitotic agents and either antimetabolites or alkylating agents produced significantly more antitumor effect than when both agents were given together [12–14]. Synchronization and recruitment mechanisms were suggested as the explanation for this potentiation of antitumor effect.

Third, the dose of VCR used in this program was lower than that used in Levitt's routine. This did not appear to interfere with the rate of response and resulted in a low incidence of neurotoxicity.

The 100% response rate of regimen in patients with HL, previously untreated with chemotherapy, with 88% CR was superior to that reported with standard CTX, VCR and PRD (COP) regimens with remission rates between 70 and 85%, and a CR rate from 35 to 50% [2, 4]. In experience previously reported from our department, the addition of other alkylating agents and of bleomycin to the COP routine did not result in better rates of either remission or survival [15]. However, recently a regimen consisting of bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP) was reported to produce 84% CR + PR (48% CR) with somewhat more strict criteria [6] for qualification of partial remission. These patients did not have prior radiation therapy and were much younger than our untreated group (median age 45 vs 70 yr). Prolonged disease-free survival of more than 3 yr with the possibility of "cure", has been reported in patients with HL with CR following completion of intensive combination chemotherapy [4, 5]. In our series, 4 patients have had prolonged remission. However, in three the chemotherapy program was not completed, indicating that prolonged remission, although dependent upon achieving a CR status, does not necessarily require prolonged courses of therapy.

Although there was good tolerance in most of the patients to these large doses of chemotherapy, the toxicity was unpredictable in some. One of the drug related deaths occurred after chemotherapy with VCR–CTX–PRD, indicating that potentiation of the antitumor effect by the time sequencing used in the study may be accompanied by potentiation of bone marrow toxicity. The extensive fibrosis of liver and lung observed in one of the responders is unique. Fibrosis as a toxic manifestation may occur after prolonged exposure to MTX [16], but there have been no reports of the simultaneous incidence of both liver and lung fibrosis with schedules utilizing large doses of MTX and LCV rescue.

The successful utilization of cell cycle dependent drugs would appear to open new aspects of therapy for histiocytic lymphoma. Intensive MTX followed by LCV has shown promise in lymphoma [17] and other tumors and is worth further investigation both alone and in combination with other drugs for therapy of this type of lymphoma. Other cell cyle dependent agents and further exploration of variations in sequencing may yield further improvements in the treatment of these diseases. The risk of increased toxicity from such intensive routines appears to be lower than expected.

This program of chemotherapy was also tested in 14 patients who previously failed during combination chemotherapy. Eleven had diffuse histiocytic and 7 had poorly differentiated diffuse lymphocytic lymphoma. Eleven patients responded with CR in 5. It was interesting that 5 of 9 of these patients who were failures to previous CTX–VCR–PRD routine given in standard doses responded to treatment with CR in 2. Among these 5 patients, three did not show response during the MTX-Ara-C phase but responded during the VCR–CTX–PRD phase, suggest-

ing that the time interval between VCR and CTX was an important factor in the restoration of the responsiveness. In addition, larger pulse doses of the CTX may have been a factor. This program offers an alternative to previously treated patients who fail to standard routines of chemotherapy [18].

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Growth and Inhibition of Mouse or Human Tumors in "Nude" (Athymic) Mice Monitored by the Extent of ^{125}I-5-Iodo-2′-Deoxyuridine (^{125}IUdR) Uptake*

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Abstract—*The effect of Ara-C or cyclophosphamide (Cy) on the growth of Lewis lung carcinoma was monitored in C57Bl/6 mice by measuring the tumor uptake of ^{125}IUdR. Strong inhibition of isotope uptake occurred* 24 hr *after drug administration. However, rapid recovery of ^{125}IUdR uptake was found in Ara-C but not in Cy-treated neoplasms. Survival of tumor-bearing mice confirmed that Cy but not Ara-C was active in producing long-term inhibition of tumor growth. Similar studies conducted with murine (Lewis lung carcinoma) or human (HeLa) tumors in athymic "nude" mice suggested that Cy was more active than Ara-C in inhibiting tumor growth* in vivo. *In further experiments the ^{125}IUdR uptake of human colon carcinoma, liposarcoma, breast carcinoma, melanoma and Burkitt lymphoma was found to be depressed by treatment with drugs (i.e. 5-fluorouracil, Me-CCNU or adriamycin) or by exposure to X-rays. It is concluded that the ^{125}IUdR uptake test could be utilized for the rapid evaluation of the antitumor activity of antineoplastic agents against human tumors growing in nude mice.*

INTRODUCTION

IN RECENT years wide interest has been focused on athymic nude mice, largely incompetent of mounting T-dependent allograft [1] or xenograft responses [2]. One of the most attractive applications of the peculiar immunobiology of the nude mouse resides in the possibility of successful xenogeneic transplantation of human tumors [2–5] for biological [6–10] and chemotherapeutic studies [11–13].

A rapid and predictive test capable of evaluating the anti tumor activity of antineoplastic agents against human tumor cells, inoculated into nude mice, would be of great help in selecting the most active drugs for clinical application, tailored to the patient bearing the tumor used for xenotransplantation. The uptake of ^{125}IUdR has been used extensively for monitoring tumor growth [14], or tumor growth inhibition by antitumor agents [15] or immune responses [16].

Therefore studies were conducted in order to test whether the same technique would be applicable to the evaluation of growth or inhibition of human tumors inoculated into nude mice. The results reported in the present communication suggest that the ^{125}IUdR uptake method could be of value for measuring the extent of growth inhibition of neoplastic cells, induced by radiation or chemotherapeutic agents in tumor-bearing nude mice.

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MATERIALS AND METHODS

Animals

Conventional inbred C57Bl/6Cr (*H*−2B),

or hybrid (C57Bl/6Cr x DBA/2Cr)F_1 (BD2F_1, $H-2^b/H-2^d$) female mice, 2–4 months old were used. BALB/c/A/BOMCr mice, ($H-2^d$ nu/nu, strain on BALB/c background, maintained in a closed but not strictly inbred colony, hereafter referred to as BALB-nu/nu), and randombred nude mice (hereafter referred to as Swiss-nu/nu) bred and maintained in a pathogen-free environment, 1–2 months old were used. Conventional mice were obtained from Dr. J. G. Mayo and Mr. C. Reeder of the Mammalian Genetics and Animal Production Section (National Cancer Institute, Bethesda, Maryland). BALB-nu/nu were bred at the Institute of Pharmacology, University of Perugia, Italy and Swiss-nu/nu were bred at the Stehlin Foundation for Cancer Research, Houston, Texas.

Tumors

Lewis lung carcinoma (LLC) of C57Bl/6 origin was passaged in C57Bl/6Cr mice by subcutaneous transplantation of tumor cells suspended in medium 199. The following human tumors were used: HBT-3 (breast carcinoma), FO No. 1 (melanoma), RA No. 1 (Burkitt Lymphoma), BA No. 1 (Liposarcoma), CR No. 1 (colon carcinoma), (CR No. 1, CR No. 8) or Swiss-nu/nu (FO (tumor line obtained after long-term passages in tissue culture in medium 199 containing 10% Fetal Bovine Serum, GIBCO). All these tumors except HeLa cells were used after several sc passages in BALB-nu/nu (CR No. 1, CR No. 8) or Swiss-nu/nu (FO No. 1, RA No. 1, BA No. 1) mice.

Drugs

Cyclophosphamide (Cy), adriamycin (ADM), cytosine arabinoside (Ara-C), 1-chloroethyl-3-4-methyl-cyclohexyl-1-nitrosurea (Me-CCNU), 5-fluorouracil (5-FU), 5-fluoro-2-deoxyuridine (FUdR) were supplied by Dr. J. B. Wood, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland.

Irradiation

Mice bearing solid tumors were shielded with lead block 10 mm thick, leaving exposed to radiation the neoplastic area. Irradiation was carried out with a Westinghouse X-ray machine (Quadrocondex; 200 kW, 15 mA, 0.25 mm copper filter and 0.55 aluminum filter, 54 cm distance, irradiation rate 137 R/min).

^{125}IUdR uptake test

Normal control or tumor-bearing mice were injected i.p. with FUdR (25 μg/mouse) to inhibit the endogenous synthesis of thymidine [14], followed 1 hr later by 1–4 μCi/mouse of ^{125}IUdR i.p. (Radiochemical Centre, Amersham, England; specific activity 8–10 μCi/mg). One day later the tumor mass or comparable mass of normal subcutaneous or muscular tissue was removed and transferred into previously weighed plastic vials and weighed again. The radioactivity was measured in a well-type crystal scintillation counter (Packard, Model 5110).

RESULTS

Conventional C57Bl/6Cr mice were inoculated subcutaneously with 2×10^5 LLC cells. On day 4, 6, 10, 11, 14 and 17 after challenge the mice received FUdR and ^{125}IUdR for performing the uptake test. The percentage of ^{125}IUdR uptake by the neoplastic tissue in the course of tumor growth is illustrated in Fig. 1. Progressive

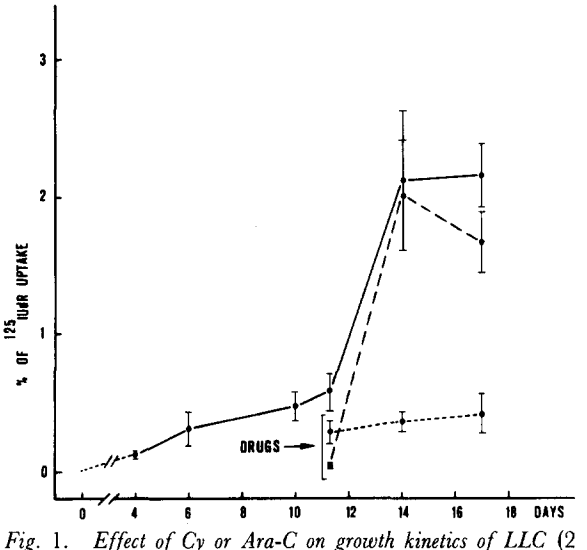

Fig. 1. Effect of Cy or Ara-C on growth kinetics of LLC (2 x 10^5 cells s.c.) in C57Bl/6 mice.

●———● *Control mice;* ●– – – –● *Mice treated with Cy;* ●— — —● *Mice treated with Ara-C.*

Each determination was performed with 7 mice/group. The first determination of ^{125}IUdR uptake after treatment with Cy (180 mg/kg) *or Ara-C* (1 g/kg) *was performed* 6 hr *after drug administration.*

The arithmetic mean ± standard error was calculated on the original count/minute.

but limited increase of isotope uptake was observed during the initial period of 11 days after LLC challenge. Marked increase occurred, from day 11 to 17 after challenge, when rapid and progressive growth of solid subcutaneous tumor was observed. Two

additional groups of mice were treated on day 11 with a single injection of Cy (180 mg/kg i.p.), a drug active in prolonging survival of LLC-bearing hosts [17], or with Ara-C (1 g/kg i.p.), extremely active in inhibiting DNA synthesis but scarcely effective in prolonging survival times of LLC-bearing mice [17]. The tumor uptake of ^{125}IUdR was initially inhibited in both Cy and Ara-C treated mice (Fig. 1). On day 14 and 17 after challenge (i.e. 3 and 6 days respectively after drug treatment), the values of tumor ^{125}IUdR uptake in Ara-C treated mice reached the levels of those of untreated controls, whereas they remained markedly low in Cy-treated hosts. The effect of Cy or Ara-C on tumor growth in terms of ^{125}IUdR uptake, tumor weight or mortality was studied in the same tumor system, as illustrated in Table 1. C57Bl/6 Cr or BD2F$_1$Cr mice were challenged sc with 2×10^5 cells of LLC and treated i.p. with Cy (200–180 mg/kg) or Ara-C (1 g/kg) on day 8. The ^{125}IUdR uptake test was carried out on day 13 or 14 after challenge (i.e. 5–6 days after drug treatment). The data confirmed the efficacy of Cy in inhibiting tumor growth as judged by significant decrease of ^{125}IUdR uptake or tumor weight, or by increase in survival time. On the contrary, marginal or no inhibitory effect on isotope uptake, tumor weight or survival time was found following Ara-C treatment.

The LLC model was used for studying tumor growth and drug-induced tumor inhibition in nude mice, as illustrated in Table 2. Successful allogeneic tumor growth occurred in $H-2^d$ nude mice as evidenced by tumor weight and tumor ^{125}IUdR uptake values on day 11. Moreover, significant inhibition of tumor growth occurred both in syngeneic conventional C57Bl/6 or allogeneic nude mice injected with Cy (180 mg/kg) on day 8 after LLC challenge. Again, marginal or no inhibition of tumor growth was found following treatment with Ara-C (1 g/kg).

Four human tumors, HeLa (2×10^6 cells), CR No. 1, CR No. 8 (tumor fragments) and BA No. 1 (10^7 cells), were inoculated subcutaneously into nude mice. When appreciable nodules appeared at the injection

Table 1. Effect of cyclophosphamide or Ara-C on ^{125}IUdR uptake by subcutaneous tumor, tumor weight and survival of mice challenged with LLC (2×10^5 cells s.c.)

Host*·	Treatment†	% ^{125}IUdR uptake by subcutaneous tumor‡ Mean ± S.E.‖	Tumor weight (g) Mean ± S.E.‖	MST§
C57Bl/6	None	1.30 ± 0.20	1.83 ± 0.25	NT¶
C57Bl/6	Cyclophosphamide	0.35 ± 0.11**	0.46 ± 0.05**	NT
C57Bl/6	Ara-C	1.74 ± 0.28	1.72 ± 0.18	NT
BD2F$_1$	None	1.53 ± 0.29	1.68 ± 0.38	27 (23–33)
BD2F$_1$	Cyclophosphamide	0.64 ± 0.29**	0.55 ± 0.14**	42 (37–56)††
BD2F$_1$	Ara-C	1.23 ± 0.25	0.95 ± 0.13	34 (29–39)

*8 C57Bl/6Cr mice per group, 16 BD2F$_1$Cr mice per group: 8 for ^{125}IUdR uptake test and 8 for survival studies.

†Cyclophosphamide (200 mg/kg i.p. in C57Bl/6 or 180 mg/kg i.p. in BD2F$_1$ mice) or Ara-C (1 g/kg i.p.) was given as a single injection on day 8 after challenge.

‡^{125}IUdR was given on day 13 (in C57Bl/6) or day 14 (BD2F$_1$) after challenge (see Materials and Methods). The % ^{125}IUdR uptake by normal subcutaneous or muscular tissue did not exceed 0.44/g of fresh tissue.

§MST, mean survival time in days, in parenthesis the range of mortality. Groups of 8 mice each, not subjected to the ^{125}IUdR uptake test.

‖Arithmetic mean ± standard error.

¶NT, not tested.

**Statistically significant difference from the value of untreated control, according to Student's *t*-test ($P<0.05$) calculated on the original counts per minute or on tumor weights.

††Statistically significant increase in survival time over survival of untreated controls ($P<0.01$, calculated according to Mann–Whitney U-test).

Table 2. Effect of cyclophosphamide or Ara-C on ^{125}IUdR uptake by subcutaneous tumor and tumor weight in conventional or nude mice challenged with LCC (2×10^5 cells s.c.)

Host*	Treatment†	% ^{125}IUdR uptake by subcutaneous tumor‡ Mean ± S.E.§	Tumor weight (g) Mean ± S.E.§
C57Bl/6	None	1.08 ± 0.35	0.67 ± 0.23
C57Bl/6	Cyclophosphamide	0.21 ± 0.03‖	0.37 ± 0.01
C57Bl/6	Ara-C	0.67 ± 0.13	0.76 ± 0.17
BALB-nu/nu	None	1.47 ± 0.31	1.56 ± 0.52
BALB-nu/nu	Cyclophosphamide	0.27 ± 0.08‖	0.73 ± 0.17
BALB-nu/nu	Ara-C	1.36 ± 0.22	1.22 ± 0.28

*5 animals per group.
†Cyclophosphamide (180 mg/kg i.p.) or Ara-C (1 g/kg i.p.) was given as a single injection on day 8 after challenge.
‡^{125}IUdR was given on day 11 after challenge (see Materials and Methods). The % ^{125}IUdR uptake by normal subcutaneous or muscle tissue did not exceed 0.05/g of fresh tissue.
§Arithmetic mean ± standard error.
‖Statistically significant difference from the value of untreated control, according to Student's *t*-test ($P < 0.05$) calculated on the original counts per minute. No statistically significant differences were found in tumor weights.

site (24–30 days after challenge), the mice were assigned randomly to control or drug-treated groups. Mice bearing HeLa cells were treated on day 24 with a single i.p. injection of Cy (180 mg/kg) or Ara-C (1 g/kg), BALB nu/mu bearing CR No. 1 colon carcinoma were treated with a single i.p. injection of 50 mg/kg of 5-FU and nude mice bearing CR No. 8 colon carcinoma received a single i.p. injection of 5-FU (50 mg/kg) or Me-CCNU (20 mg/kg). Swiss nu/nu bearing BA No. 1 liposarcoma were treated with a single i.p. injection of 6 or 12 mg/kg of adriamycin (Table 3). The ^{125}IUdR test was performed two (BA No. 1) or three (HeLa, CR No. 1, CR No. 8) days after drug treatment. The results (Table 3) show that marked inhibition of HeLa growth was afforded by Cy but not by Ara-C. Adriamycin seems to provide growth inhibition of BA No. 1 liposarcoma. Moreover, 5-FU and Me-CCNU seem to be active in reducing the growth of CR No. 8, although the data were not statistically significant possibly as a result of the low number of observations.

In further preliminary studies, nude mice were inoculated s.c. with 10^7 cells of HBT-3, FO No. 1 or RA No. 1 tumors, on four sites located in the front or in the back of the animal.

Two of the four tumors of each animal were irradiated, and the ^{125}IUdR uptake test was performed 19 hr after irradiation.

The results of the experiment (Table 4) showed that substantial inhibition of tumor growth was found after exposure to 500–1200 R of HBT No. 3 and FO No. 1 melanoma tumors, or RA No. 1 Burkitt lymphoma.

DISCUSSION

The results reported in the present communication show the following: (a) the growth of a murine solid tumor can be monitored by the extent of ^{125}IUdR uptake by neoplastic tissue either in conventional or nude mice; (b) the growth of human tumors in nude mice can be monitored using the same technique; (c) drug or radiation-induced inhibition of murine or human tumor cells can be evaluated by measuring the reduction of ^{125}IUdR incorporation, in conventional and in nude mice as well. The labeled compound ^{125}IUdR is an analog of thymidine, and is incorporated into DNA in place of thymidine during the S phase of the cell cycle [14]. Therefore the evaluation of ^{125}IUdR uptake can be considered a rather accurate measure of cell proliferation [14]. The data obtained in conventional mice inoculated with LLC are in agreement

Table 3. Effect of antineoplastic drugs on ^{125}IUdR uptake by subcutaneous tumor, and tumor weight in nude mice challenged with human tumors s.c.

Host	Tumor: No. of mice	Tumor: Type‡	Tumor: No. cells injected s.c.	Treatment*: Drug	Treatment*: Dose mg/kg i.p.	% ^{125}IUdR† uptake by subcutaneous tumor Mean ± S.E.§	Tumor weight(g) Mean ± S.E.
BALB-nu/nu	5	HeLa	2×10^6	none	—	0.39 ± 0.10	0.38 ± 0.10
BALB-nu/nu	5	HeLa	2×10^6	Cyclophosphamide	180	0.05 ± 0.01**	0.36 ± 0.13
BALB-nu/nu	5	HeLa	2×10^6	Ara-C	1000	0.27 ± 0.06	0.31 ± 0.08
BALB-nu/nu	2	CR No. 1	tf‖	—	—	0.16 ± 0.01	0.15 ± 0.03
BALB-nu/nu	2	CR No. 1	tf	5-FU	50	0.07 ± 0.02	0.11 ± 0.04
BALB-nu/nu	4	CR No. 8	tf	—	—	0.14 ± 0.08	0.04 ± 0.05
BALB-nu/nu	4	CR No. 8	tf	5-FU	50	0.07 ± 0.05	0.08 ± 0.03
BALB-nu/nu	4	CR No. 8	tf	Me-CCNU	20	0.04 ± 0.01	0.09 ± 0.03
Swiss-nu/nu	2	BA No. 1	10^7	none	—	0.16 ± 0.05	NT¶
Swiss-nu/nu	2	BA No. 1	10^7	ADM	6	0.16 ± 0.6	NT
Swiss-nu/nu	2	BA No. 1	10^7	ADM	12	0.07 ± 0.02	NT

*24–30 days after tumor challenge. Drugs were given as a single i.p. injection.
†^{125}IUdR was given 48 hr (in mice bearing BA No. 1) or 72 hr after drug treatment (see Materials and Methods). The % ^{125}IUdR uptake by normal subcutaneous or muscle tissue did not exceed 0.014/g of fresh tissue.
‡CR No. 1 colon carcinoma; CR No. 8 colon carcinoma; BA No. 1 liposarcoma.
§Arithmetic mean ± standard error.
‖tf, tumor fragment 3 × 3 mm s.c.
¶NT, not tested. The weight of the tumors was approximately 1 g in all control or ADM-treated groups.
**Statistically significant difference from the value of untreated control, according to Student's *t*-test ($P<0.01$) calculated on the original counts/minute. No statistically significant differences were found in tumor weights.

Table 4. Effect of radiation on ^{125}IUdR uptake by subcutaneous tumor, and tumor weight in nude mice challenged with human tumors s.c.

Tumor*: Type	Tumor*: Position†	Radiation‡	% ^{125}IUdR uptake§ by subcutaneous tumor Mean ± S.E.	Tumor weight (g) Mean ± S.E.	% Inhibition¶
HBT No. 3	Right and left front	—	0.055 ± 0.021	0.165 ± 0.005	—
	Right and left back	800 rad	0.011 ± 0.0001	0.105 ± 0.005	80
	Right and left front	—	0.025 ± 0.003	0.300 ± 0.008	—
	Right and left back	1200 rad	0.002 ± 0.0004**	0.235 ± 0.025	92
FO No. 1	Right and left front	—	0.029 ± 0.011	0.147 ± 0.007	—
	Right and left back	1200 rad	0.0009 ± 0.0001	0.040 ± 0.0001	97
RA No. 1	Right and left front	—	0.008 ± 0.001	0.208 ± 0.031	—
	Right and left back	500 rad	0.002 ± 0.0001**	0.188 ± 0.010	70

*HBT No. 3 Human breast tumor; FO No. 1 Melanoma; RA No. 1 Burkitt lymphoma.
†Each cell line was injected in four areas of a Swiss-nu/nu (right and left front, right and left back).
‡When the tumors grew to 5 × 5 × 5 mm, the front part of the animal was covered with a heavy, thick lead block and the back part was radiated at the doses reported in the table.
§^{125}IUdR (4 μCi/mouse) was given 18 hr after radiation treatment. The % ^{125}IUdR uptake by normal subcutaneous or muscular tissue did not exceed 0.004/g of fresh tissue.
‖Arithmetic mean ± standard error.
¶% Inhibition refers to the values of % ^{125}IUdR uptake.
**Statistically significant difference from the value of untreated control, according to Student's *t*-test ($P<0.05$) calculated on the original counts/min.

with previous studies in a similar experimental model [17]. The growth of LLC was comparable in C57Bl/6 and in allogeneic nude mice (Table 2), as judged by the ^{125}IUdR uptake values and tumor weights, which were even greater in allogeneic thymus-less than in syngeneic recipients. This is in agreement with previous findings showing lack of T-dependent allograft responses in nude mice [18]. Inhibition of ^{125}IUdR uptake by murine tumors seems to be a rather sensitive index of host-anti-graft

immune response either of T-dependent or T-independent origin [16, 19]. Therefore it can be suggested that LLC tumor cells (of $H-2^b$ origin) do not express tumor-associated antigens capable of eliciting non-T dependent transplantation resistance in nude mice, similar to that detectable against $H-2^b$ lymphomas [19]. This can be explained on the basis that tissues of hematopoietic origin possess Hh (Hematopoietic-histocompatibility) antigens capable of eliciting T-independent responses [20–22], whereas some tissues of non-hematopoietic origin do not express detectable amounts of such antigens (Cudkowicz, personal communication).

Successful growth of xenogeneic human tumors can be attained in nude mice, as previously reported [2–5]. The possibility of monitoring the extent of neoplastic growth by ^{125}IUdR uptake seems to be confirmed by data of Tables 3 and 4, showing that significant uptake of the isotope occurred in the human tumors inoculated into nude recipient mice.

The data concerning the effect of drug treatment on ^{125}IUdR uptake (Fig. 1) or tumor weight (Tables 1–2) or survival of tumor-bearing hosts (Table 1) lead to several hypotheses. Antineoplastic agents, either capable of marked inhibition of tumor growth such as cyclophosphamide, or strong inhibitors of DNA polimerase, such as Ara-C [23], but scarcely active on LLC in terms of host's survival [17], provoked almost complete block of ^{125}IUdR uptake within a few hours of drug administration (Fig. 1). Nevertheless a rapid recovery of ^{125}IUdR uptake occurred in LLC-bearing mice treated with the relatively inactive Ara-C, whereas long-lasting inhibition of DNA synthesis occurred in LLC of mice treated with an active drug, such as cyclophosphamide. Therefore the differential timing in the recovery of ^{125}IUdR uptake could be related to the sensitivity of neoplastic cells to the antitumor effects of chemotherapeutic agents.

The results obtained with HeLa tumor cells growing in nude mice (Table 3) would suggest that the tumor is sensitive to Cy rather than to Ara-C treatment, although no parallel studies on survival times have been made and no reduction of tumor weight was found in Cy-treated hosts, in which significant inhibition of ^{125}IUdR uptake occurred. However, further studies (Rivosecchi Merletti P., unpublished data) suggested that treatment with Cy but not with Ara-C was capable of producing limited inhibition of HeLa cells in nude mice, without affecting tumor size in a short-term range. The growth of liposarcoma BA No. 1 or colon carcinomas CR No. 1 and CR No. 8 seemed to be inhibited by ADM or 5-FU and Me-CCNU respectively. Nevertheless the experimental data did not reach the level required for statistical significance. This could be ascribed to the limited number of observations and to the high variability of the ^{125}IUdR uptake in control mice. Therefore higher number of animals will be required for further investigation.

Limited growth rate was found in human tumors in the experiments of localized irradiation (Table 4), as evidenced by the low ^{125}IUdR uptake detectable in non-irradiated neoplastic nodules. In irradiated tumors, reduction of tumor weight was generally found shortly after irradiation. However the decline of ^{125}IUdR uptake was much more pronounced than the decrease of tumor mass. In this case we cannot rule out that the extent of isotope uptake was a more sensitive and specific parameter than weight change for evaluating the cytotoxic and/or cytostatic effects of X-rays [24]. Nevertheless the results of this study are quite preliminary, since no kinetics of isotope uptake after irradiation have been made.

In conclusion the data obtained in the present investigation suggest that the evaluation of the uptake of ^{125}IUdR could be used as a sensitive method to monitor the growth and drug-induced growth inhibition of experimental or human tumors inoculated into nude mice. Further studies are required in order to assess the actual value of the method for predicting the efficacy of antineoplastic agents against selected human tumors.

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Analysis of Synthetic RNA-Directed DNA Polymerase Activities in the Spleen of a Rat with Myelogenous Leukemia Induced by *N*-Nitroso-*N*-Butylurea*

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Abstract—*DNA polymerase activities from the spleen of an individual rat with myelogenous leukemia induced by* N-*nitroso*-N-*butylurea were examined in view of their possible association with RNA tumor viruses.*

Viral-like DNA polymerase was not detected when DNA polymerase activities of the cells were analyzed as follows:

(1) *Phosphocellulose chromatography of the extract from leukemic spleen gave a single peak of poly(rA)·(dT)*$_{12-18}$ *directed activity which coincided with that of DNA polymerase γ obtained from normal rat spleen, whereas the chromatography of the extract from virus-producing cultured R*35 *rat mammary tumor cells or from those viruses themselves gave a unique peak of activity different from that of DNA polymerase γ. These viruses appeared to be one of the rat endogenous viruses similar to the R*35 *virus.*

(2) *The peak from the leukemic spleen showed several enzymatic properties similar to those of DNA polymerase γ obtained from normal rat spleen, but different from those of the R*35 *viral DNA polymerase.*

(3) *The peak from the leukemic spleen responded to poly(rCm)·(dG)*$_{12-18}$, *which is reported to be specific for viral reverse transcriptase. However, DNA polymerase γ fraction from normal rat spleen also responded to the template-primer to the same degree.*

(4) *Postmitochondrial fractions from the leukemic spleen gave poly(rA)·(dT)*$_{12-18}$*-directed activity sedimented at a density of* 1.17 g/cm^3 *on sucrose. However, addition of nonionic detergent was not required for the activity. In the case of our R*35*-like viruses banded at a density of* 1.16 g/cm^3, *the detergent was required for detection of the activity.*

INTRODUCTION

RNA VIRUSES of type C morphology are etiologically associated with leukemia, lymphoma, and solid tumors of a variety of mammalian species, including mouse, rat, hamster and cat.

On the other hand, a number of chemicals or radiation is known to induce tumors, including leukemia, in animal species [1]. The possibility that viruses are involved in such chemical or radiation carcinogenesis has been raised and discussed. Chemical reagents or ionizing radiation may trigger the activation of the hitherto latent viruses, which then could acquire a pathogenic potential, causing the development of tumors [1]. In fact, some chemicals, for example, bromodeoxyuridine, iododeoxyuridine, or cycloheximide are known to activate the endogenous RNA viruses in the mammalian cells *in vitro* [2, 3].

Oral administration of *N*-nitroso-*N*-butylurea (NBU) to the rat induces myelogenous leukemia in high frequency [4].

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This myelogenous leukemia in rats appears to be more comparable to the human situation [4] than is the thymic lymphosarcoma of mice induced by NBU [5, 6]. No rat leukemia is known to be associated with virus, however [4]. With the leukemia rat, no successful attempt has thus far been made (1) to observe type C virus particles by electron microscope, (2) to prepare oncogenic cell-free filtrates, *or* (3) to detect group-specific cell surface antigen from the leukemia cells [4, 7]. Libertini and Adams, however, have suggested a possible involvement of virus in chemical carcinogen-induced rat mammary tumors through their analysis of RNA-directed DNA polymerase activities [8].

In the present study, we examined the activity of reverse transcriptase as a marker of virus association in the individual leukemic rat, using cell-free extracts of the enlarged spleen, in which leukemic cells were fully proliferated. We used poly(rA)·$(dT)_{12-18}$ as template-primer to detect the activity of reverse transcriptase in the cell extracts. However, since the cellular DNA polymerase γ also responds to poly(rA)·$(dT)_{12-18}$ very efficiently, the activities of the two enzymes must be discriminated from each other by some other means. Column chromatography, molecular weight determination, and comparison of several enzymatic properties were carried out for that purpose.

The results show that any unique DNA polymerase activity different from cellular DNA polymerase γ was not detectable from the spleen of a rat with leukemia, under the conditions where reverse transcriptase activity was detectable in the virus-producing control rat cells.

MATERIALS AND METHODS

Rats with myelogenous leukemia

To obtain rats with myelogenous leukemia, the methods of Odashima [4] were followed; female Sprague–Dawley rats at the age of 11 weeks (purchased from Clea Japan) were continuously given 0.04% (w/v) *N*-nitroso-*N*-butylurea (NBU) solution as their drinking water. The NBU solution was freshly prepared every day. After 20 weeks, the rats were then kept on the basic diet alone until they were sacrificed for autopsy. Spleen was removed from the rats and kept frozen at −80°C until used. The leukemic rats were characterized by hepatosplenomegalia and by, in most cases, a high level of white blood cell count in the peripheral blood. The spleen weighed 2.1 g on the average (0.5 g for normal rats) and normal spleen architecture was seldom seen due to the extensive metastatic infiltration of leukemic cells.

R35 rat mammary tumor cells

63-sp-6-Cl-8 [9], a subcloned culture of R35 rat mammary tumor cells which was established originally from transplantable spontaneous Haggins mammary fibroadenoma A [10] was grown at 37°C in GIBCO Nutrient mixture F-12 medium, supplemented with 10% fetal calf serum and 60 mg/l Kanamycin, in 250 ml plastic tissue culture flasks (Falcon Plastics). This culture line was established from original R35 tumors frozen in liquid nitrogen in 1963 and showed high reverse transcriptase titers [9]. These viruses were found to be one of the rat endogenous viruses [Y. Ikawa, unpublished observations]. They have been called R35 virus [11] and the capacity of this virus to transform cultured mammary epithelial cells was discussed [12]. Each flask was seeded with 5×10^4 viable cells per ml. The cell cultures were developed as confluent monolayers. The cells were collected by scraping them off from the bottom of flasks by using a rubber policeman. The cells were washed 2 times with the above medium but minus serum by centrifugation. The cell pellet was stored at −80°C until used.

R35 virus particles

R35 viruses were recovered from the above culture. The harvested culture medium was centrifuged first at $16{,}000 \times g$ for 15 min. The supernatant was then centrifuged at $106{,}000 \times g$ for 60 min. The pellet was washed once with a buffer containing 0.1 M NaCl–10 mM Tris–HCl (pH 7.5)–1 mM EDTA by centrifugation. The virus pellet was suspended in the above buffer and stored at 0°C until used.

Preparation of crude extracts for phosphocellulose column chromatography

Spleen extracts were prepared by a modification of the procedure described by Yang *et al.* [13]. All procedures were performed at 0°–4°C unless otherwise specified. Spleen from individual rats was separately thawed, minced, suspended in 4 vol of a buffer containing 0.4 M sucrose–50 mM Tris–HCl (pH 8.0)–50 Mm KCl–4 mM magnesium acetate–2 mM dithiothreitol (DTT), and

mechanically disrupted in a Potter Elvjheim homogenizer with 5 strokes. The homogenate was centrifuged at $1000 \times g$ for 10 min. Nonidet P40 (nonionic detergent) and KCl were added to the supernatant to give final concentrations of 0.5% (w/v) and 0.5 M, respectively. It was incubated at 37°C for 10 min. The pellet was suspended in a buffer containing 0.5% (w/v) Nonidet P40–50 mM Tris–HCl (pH 8.0)–0.5 M KCl–4 mM magnesium acetate–2 mM DTT-15% (v/v) glycerol, and incubated at 37°C for 10 min. The cytoplasm and nuclear fractions were separately centrifuged at $164,000 \times g$ for 90 min. The two supernatants were combined and dialyzed against buffer A containing 50 mM imidazol (pH 6.5)–0.1 M KCl–1 mM DTT–20% (v/v) glycerol [14] and then chromatographed directly on a column of phosphocellulose.

R35 mammary tumor cells were thawed, suspended in 4 vol of a buffer containing 0.5 M KCl–10 mM Tris–HCl (pH 7.5)–1 mM EDTA–2 mM DTT–4 mM magnesium acetate–0.5% (w/v) Nonidet P40–20% (v/v) glycerol, and sonicated by a Branson sonifier in iced-water until more than 98% of the cells were disrupted. The homogenate was frozen and thawed twice, centrifuged first at $100 \times g$ for 10 min., and then at $164,000 \times g$ for 90 min. The supernatant was dialyzed against buffer A (see above) and then chromatographed directly on a column of phosphocellulose.

R35 virus particles were suspended in a buffer containing 5% (w/v) Nonidet P40–0.7 M KCl% 10 mM Tris–HCl (pH 7.5)–1 mM EDTA–10% (v/v) glycerol–1 mM DTT, and kept overnight on iced-water in order to solubilize the enzyme from the virus particles [15]. The suspension was centrifuged at $106,000 \times g$ for 60 min and the supernatant dialyzed against buffer A and subjected to phosphocellulose chromatography.

Preparation of postmitochondrial fractions for sucrose density gradient analysis

Spleen was mechanically disrupted in a Potter–Elvjheim homogenizer in the same manner as described above and subjected to sequential sedimentation by differential centrifugation but in the absence of Nonidet P40, KCl, and magnesium acetate [16]. The postmitochondrial sediments obtained by a final centrifugation at $100,000 \times g$ for 60 min were suspended in phosphate-buffered saline (pH 7.2) and subjected to centrifugation analysis on a sucrose density gradient [16].

Phosphocellulose column chromatography

Phosphocellulose (Whatman P11) was washed with 0.5 M NaOH followed by 0.5 M HCl. The pH was brought to 6.5 by addition of solid Tris base [14]. A column was packed, using only gravity, and equilibrated with approx. 30 column vol of buffer A. The crude extracts from spleen, R35 mammary tumor cells, or R35 virus as described above were loaded onto the column and the elution was performed first with buffer A and then with a linear KCl gradient in buffer A.

Sucrose density gradient centrifugation

The postmitochondrial fractions from spleen or R35 virus fraction, which were obtained as described above, were layered onto an 11.6 ml 20–70% sucrose gradient in phosphate-buffered saline (pH 7.2) and centrifuged in a Spinco SW41 rotor for 16–18 hr at $150,000 \times g$ [16].

Fractions were collected and assayed for DNA polymerase activity. Densities of the fractions were calculated from refractive index measurements.

Glycerol gradient centrifugation

A portion of DNA polymerase fractions from phosphocellulose was dialyzed against a buffer containing 5% (v/v) glycerol–50 mM Tris–HCl (pH 7.5)–0.1 M KCl–1 mM EDTA, and layered over a 4.8 ml of 10–30% (v/v) linear glycerol gradient containing 50 mM Tris–HCl (pH 7.5)–0.25 M KCl–1 mM DTT–0.1 mM EDTA [17]. The gradient was centrifuged for 15 hr at 1°C at 60,000 rev/min in a Hitachi RPS65T rotor. Fractions of approx. 0.25 ml were collected from bottom of the gradient. Ten microlitre aliquots of each fraction were assayed for DNA polymerase activity in the presence of poly(rA)·$(dT)_{12-18}$ as template-primer. Sedimentation coefficient and molecular weight of the DNA polymerases were estimated by the method of Martin and Ames [18] using the protein standards: bovine liver catalase [11.3s, molecular weight (mol. wt) of 245,000], yeast alcohol dehydrogenase [7.6S, mol.wt of 150,000] and egg white lysozyme [2.1S, mol.wt of 17,200].

DNA polymerase assay

Two types of reaction mixture were used

as follows. They were essentially the same as those used by Yang *et al.* [13].

With synthetic template-primers. The reaction mixture (final volume of 50 μl) contained: 8.5 μg of bovine plasma albumin, 25 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.5 mM $MnCl_2$, 2 mM DTT, 2 μCi of [^{3}H]dTTP -0.01 mM) or [^{3}H]dGTP (0.01 mM) and 2 μg of synthetic template-primer as specified.

With activated calf thymus DNA. The reaction mixture (final volume of 50 μl) contained; 8.5 μg of bovine plasma albumin, 25 mM Tris–HCl (pH 8.0), 40 mM KCl, 10 mM magnesium acetate, 2 mM DTT, 2 μCi of [^{3}H]dTTP (0.01 mM), other three deoxyribonucleoside triphosphates at 0.25 mM each and 10 μg of activated calf thymus DNA.

In both assay procedures, enzyme fraction was added to initiate the reaction, and the incubation was for 30 min at 37°C. Aliquots were withdrawn and the acid-insoluble fraction was precipitated on Whatman GF/C glass filters in cold 5% trichloroacetic acid–10 mM sodium pyrophosphate, washed 3 times with cold 5% trichloroacetic acid, once with ether-ethanol, and then once with ether. The filters were air-dried, and the radioactivity retained on them was measured in a Searle Analytic scintilation counter.

Preparation of synthetic template-primers

To prepare poly[rA)·$(dT)_{12-18}$, poly(rA) and oligo (dT) were mixed in 0.05 M Tris–HCl (pH 7.5) in a ratio of 4 to 1 to give a final concentration of 20 A_{260nm} units/ml. The mixture was heated for 10 min at 50°C followed by slow cooling and stored at −20°C until used. Poly(rCm)·$(dG)_{12-18}$ was prepared in the same manner as above except that heating was 80°C instead of 50°C.

Preparation of activated calf thymus DNA

Native calf thymus DNA (2.5 mg/ml) was digested with deoxyribonuclease from beef pancreas (DNase 1) to bring about 25% acid soluble ultraviolet absorbing materials. The procedures followed were Schlabach *et al.* [19].

Others

N-nitroso-*N*-butylurea was the product of Izumi Kagaku Yakuhin Co. Ltd. [^{3}H]dTTP (15–30 Ci/mmole) and [^{3}H]dGTP (10–20 Ci/mmole) were obtained from the Radiochemical Centre, Amersham. Poly(rA), poly(rCm), $(dT)_{12-18}$, $(dG)_{12-18}$, dATP, dTTP, dCTP and dGTP were obtained from P-L Biochemicals Inc. Calf thymus DNA, bovine liver catalase, yeast alcohol dehydrogenase, egg white lysozyme and *N*-ethylmaleimide were obtained from Sigma Chemical Co. Phosphocellulose (P11) was obtained from Whatman Biochemicals Ltd. DNase I and reverse transcriptase (AMV) were purchased from Worthington Biochemical Corp. and Boehringer Mannheim GmbH, respectively. DNA-cellulose and DNA polymerase γ from mouse myeloma cells were kind gifts of Dr. Matsukage of the Aichi Cancer Center Research Institute [17].

RESULTS

The aim of the present study was to examine whether RNA-directed DNA polymerase (reverse transcriptase) of RNA tumor virus is detectable in a rat with myelogenous leukemia which was induced by oral administration of *N*-nitroso-*N*-butylurea (NBU). No rat leukemia is so far known to be associated with virus [4]. Two kinds of approach were carried out for that purpose. One was to separate upon chromatography [14, 20] reverse transcriptase from cellular DNA polymerases. The other approach was to locate the DNA polymerase activity of the leukemic cell extract by centrifugation on a sucrose density gradient in which reverse transcriptase activity banded on the gradient at a density range characteristic of the virus particles [16].

Phosphocellulose column chromatography

Figure 1(a–d) exemplify the elution profiles of DNA polymerase activities on phosphocellulose column chromatography: crude extracts were obtained from a portion of the enlarged spleen of the individual rat with leukemia as described in Materials and Methods and directly chromatographed on the column. As control, extracts of spleen from a normal rat, the cultured R35 mammary tumor cells which continuously produced endogenous type C viruses [9] and the R35 virus particles were analyzed, respectively. To distinguish the activity of reverse transcriptase from that of cellular DNA polymerase γ, two kinds of template-primer were used for the assay, i.e. poly(rA)·$(dT)_{12-18}$ or poly(rCm)·$(dG)_{12-18}$; both enzymes respond to poly(rA)·$(dT)_{12-18}$, whereas only the viral enzyme is reported to respond to poly(rCm)·$(dG)_{12-18}$ [20, 21]. Activated DNA was also used as template-primer to locate the activity of DNA polymerase α and β [22].

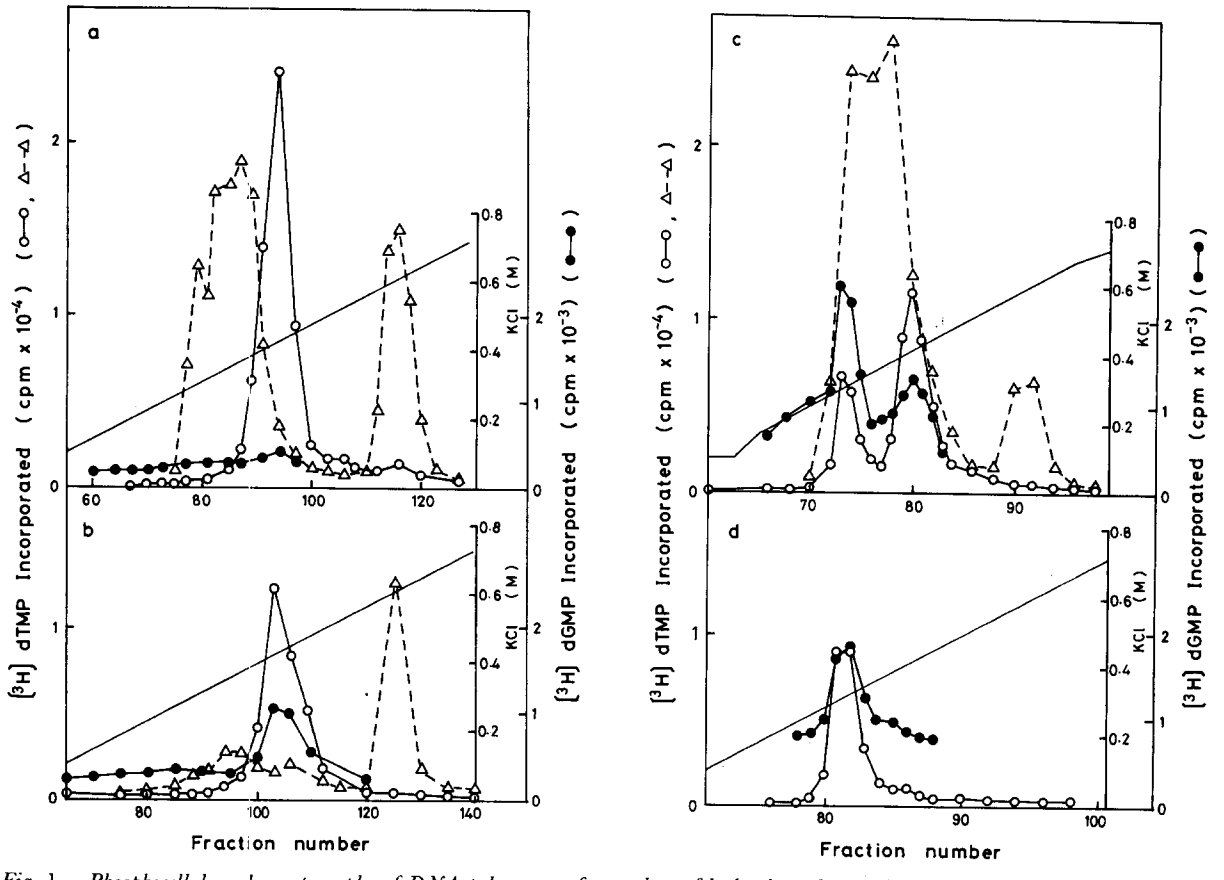

Fig. 1. Phosphocellulose chromatography of DNA polymerases from spleen of leukemia and normal rats and from R35 mammary tumor cells. Preparation of the crude extracts, column chromatography, and assay conditions for DNA polymerase activities were detailed under Materials and Methods. (a) Extracts from 1.9 g *parts of a rat spleen with NBU-induced leukemia were applied to a column* (1.5 × 23 cm) *of phosphocellulose. After a* 150 ml *wash with buffer A (see Materials and Methods), elution was carried out with* 300 ml *linear gradient of* KCl *from* 0.1 M *to* 0.8 M *at a flow rate of* 7 ml/hr. *Fractions of* 3 ml *were collected and* 5 μl *aliquots were assayed for DNA polymerase activities with poly(rA)·(dT)$_{12-18}$,* (○——○); *poly(rCm)·(dG)$_{12-18}$,* (●——●); *and activated DNA,* (△——△) *as template-primers, (b) Extracts from* 1.8 g *spleens of four normal rats together were chromatographed as in (a). (c) Extracts from* 0.5 g *of R35 rat mammary tumor cells were applied to a column* (0.8 × 20 cm) *of phosphocellulose. After a* 80 ml *wash with buffer A, elution was carried out with* 160 KCl *linear gradient from* 0.1 *to* 0.8 M *at a flow rate of* 3 ml/hr. *Fractions of* 1.5 ml *were collected and* 5 μl *aliquots were assayed for DNA polymerase activities. (d) Extract from R35 virus particles were applied to a column* (0.5 × 12 cm) *of phosphocellulose. After a* 20 ml *wash with buffer A, elution was carried out with* 20 ml KCl *linear gradient from* 0.1 M *to* 0.8 M *at a flow rate of* 1 ml/hr. *Fractions of* 0.5 ml *were collected and* 5 μl *aliquots were assayed for DNA polymerase activities.*

In the case of R35 mammary tumor cells (Fig. 1c), the activity with poly(rA)·(dT)$_{12-18}$ gave rise to two peaks; one at a KCl concentration of 0.31 M and the other one at 0.42 M. The former peak seems to represent the viral enzyme, since, (1) it coincided with the activity peak assayed with poly(rCm)·(dG)$_{12-18}$ and (2) the DNA polymerase activity from extracts of R35 virus particles eluted at the same KCl concentration (Fig. 1d). The latter peak may represent DNA polymerase γ, since it was present in all cases (Figs. 1a, b and c, respectively). It is also consistent with the data reported for other mammalian species [14, 20]. It is interesting that the latter peak also coincided with the activity directed by poly(rCm)·(dG)$_{12-18}$. Further chromatography on the DNA-cellulose column [17] of the latter peak fractions did not separate the activity directed by poly(rCm)·(dG)$_{12-18}$ from that directed by poly(rA)·(dT)$_{12-18}$ [data not shown]. It should be mentioned that these chromatographic tests were possible with 0.5 g parts of spleen or 0.3 g cultured cells and with a column of smaller size. With this smaller column, each test with five other rats with myelogenous leukemia and five normal rats gave results essentially identical to those shown, respectively, in Figs. 1(a) and (b). However, in Fig. 1(b) we used spleens from four normal rats together because of lower activities for normal rat spleen as compared to that of leukemic rat spleen. It is also noted that the extent of the enzyme activity directed by poly(rCm)·

$(dG)_{12-18}$ varied from time to time and from preparation to preparation for undetermined reasons (for examples, see the difference between the values in Figs. 1(a) and (b) and also in Experiments 1 and 2 in Table 1).

directed by each of the above two template-primers were sedimented at a density of around 1.17 g/cm^3 and also at the top of the gradient. The density of 1.16 g/cm^3 has been reported to be characteristic of RNA tumor viruses [23]. As controls, spleen from

Table 1. *Activity directed by poly(rCm)$(dG)_{12-18}$ relative to that by poly(rA)·$(dT)_{12-18}$*

Enzymes*	Template-primer poly(rCm)·$(dG)_{12-18}$ Incorporation (pmole) dGMP	Template-primer poly(rA)·$(dT)_{12-18}$ Incorporation (pmole) dTMP	Ratio of incorporation dGMP/dTMP
Experiment 1			
R35 virus	6.08	7.21	0.84
MTC PC-I	3.38	3.73	0.91
MTC PC-II	6.08	20.9	0.29
L spleen PC	12.3	45.1	0.27
N spleen PC	2.44	17.9	0.14
Mouse myeloma DNA polymerase γ	1.14	91.5	0.016
Experiment 2			
R35 virus	1.13	7.80	0.14
L spleen SDG	0.13	2.52	0.05
L spleen SDG-top	0.42	4.74	0.09
Mouse myeloma DNA polymerase γ	0.13	16.5	<0.01
AMV	0.91	13.6	0.73

*R35 virus refers to solubilized enzyme fraction after phosphocellulose chromatography (see Fig. 1d); MTC PC-I and MTC PC-II refer to pooled fractions from the 1st peak and the 2nd peak, corresponding to the viral enzyme and DNA polymerase γ, respectively, from phosphocellulose chromatography eluates of extracts from R35 mammary tumor cells (see Fig. 1c); L spleen PC and N spleen PC refer to pooled fractions from the peak corresponding to DNA polymerase γ obtained by phosphocellulose chromatography of the extracts from leukemic spleen and from normal spleen, respectively (see Figs. 1a and 1b); L spleen SDG and SDG-top refer to the fractions sedimented at a density of 1.17 g/cm^3 and at the top of the surcrose gradient, respectively, when extracts from leukemic spleen were centrifuged (see Fig. 2a); assay conditions for DNA polymerase see Materials and Methods.

Sucrose density gradient analysis

Postmitochondrial fractions were prepared from a portion of the enlarged spleen of each rat with leukemia. They were subjected to sedimentation on sucrose as described in Materials and Methods. After centrifugation the materials were fractionated and assayed for DNA polymerase activity using poly(rA)·$(dT)_{12-18}$ or activated calf thymus DNA as template-primer. Figure 2(a) shows the results obtained in the case of a rat with the leukemia. The enzyme activities a normal rats (Fig. 2b) and R35 virus particles (Fig. 2c) were analyzed in the same manner.

As for the DNA polymerase activities sedimented at a density of around 1.17 g/cm^3, the activity directed by poly(rA)·$(dT)_{12-18}$ was notable only in the case of rats with leukemia (5 out of 6 animals examined) but not in the case of normal rats. One may then assume that the activity detected at the density of 1.17 g/cm^3 is associated with the virus. From the following results,

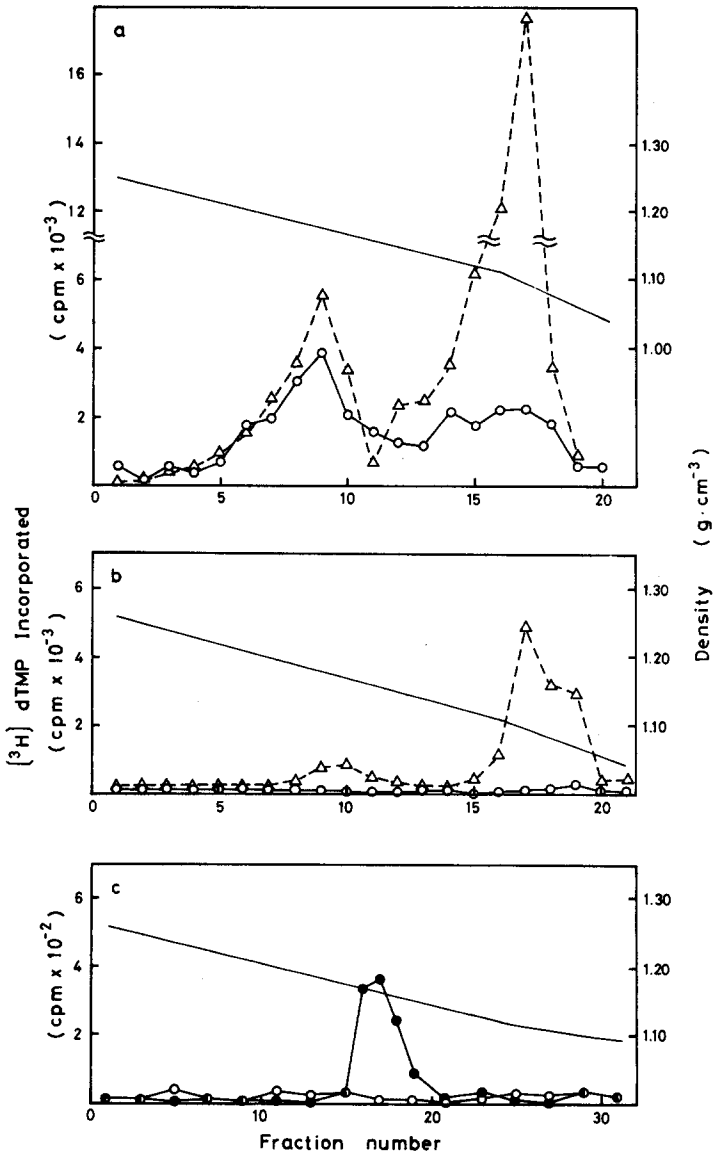

Fig. 2. Sedimentation analyses on sucrose of postmitochondrial fractions from spleen of a leukemia rat and of a normal rat and from R35 virus particles. (a) Postmitochondrial fraction from 0.4 g *of spleen of a leukemic rat was subjected to sedimentation on a sucrose density gradient. Preparation of postmitochondrial fractions and conditions for the centrifugation analysis were described under Materials and Methods. Fractions of* 10 *drops were collected and* 10 µl *aliquots were assayed for DNA polymerase activities with poly(rA)·(dT)$_{12-18}$,* (○——○); *and activated calf thymus DNA,* (△——△) *in the absence of Nonidet P40 as described in Materials and Methods. (b) Postmitochondrial fraction from* 0.4 g *of spleen of a normal rat was analysed as in (a). (c) R35 virus particles (for preparation see Materials and Methods) were subjected to sedimentation as in (a) but the assay was carried out in the presence* (●——●) *and in the absence* (○——○) *of* 0.7% w/v) *Nonidet P40, using* 20 µl *aliquots for the assay.*

however, we rather conclude that it is of cellular DNA polymerase γ: (1) The assay for the DNA polymerase activities described above was carried out in the absence of detergent, which is usually required for detection of the activities associated with virus particles [24, 25]. In fact, Nonidet P40 was required for the activity directed by Poly(rA)·(dT)$_{12-18}$ in the case of R35 virus (Fig. 2c). (2) The incorporation of dGMP directed by poly(rCm)·(dG)$_{12-18}$ was tested in the fractions of the sucrose density gradient

in the case of a rat with leukemia as shown in Table 1 (designated as Leukemic spleen SDG). The extent of the incorporation relative to that of dTMP directed by poly(rA)·$(dT)_{12-18}$ was not as prominent as that in the fractions from R35 virus particles. It should be mentioned that an attempt to visualize virus particles by an electron microscope with the negative staining method in the materials banded at a density of 1.17 g/cm^3 was without success in the case of the rat with leukemia (Fig. 2a).

Enzymatic properties

In order to further confirm that the poly(rA)·$(dT)_{12-18}$-directed DNA polymerase activity in the leukemic cells is identical with that of DNA polymerase γ, optimum assay conditions for the activity of the phosphocellulose peak from leukemic spleen was compared with that of DNA polymerase γ peak from normal rat spleen or for reverse transcriptase peak from R35 rat mammary tumor cells or from R35 virus particles; these peaks used were all obtained after phosphocellulose chromatography [Figs. 1(a–b) and legend to Table 1]. The results were as follows.

Effect of salt. Table 2 shows the optimum KCl concentrations for the activity of each peak fraction when poly(rA)·$(dT)_{12-18}$ or poly(rCm)·$(dG)_{12-18}$ was used as template-primer. Clearly, optimum KCl concentration for the activity of the peak fraction from leukemia spleen is the same as that for the DNA polymerase γ fractions obtained from spleen of normal rat, but different from that of the viral enzyme fractions either from R35 mammary tumor cells or from R35 virus particles.

Table 2. Optimal KCl *concentrations*

Enzymes	Optimum KCl concentration (mM)	
	Template-primer	
	poly(rCm)·$(dG)_{12-18}$	poly(rA)·$(dT)_{12-18}$
R35 virus	40	70
MTC PC-I	40	70
L spleen PC	5	130
N spleen PC	5	150

For identity of the enzymes see legend to Table 1.
The reaction conditions are specified in Materials and Methods.

Effect of divalent cations. Table 3 shows the optimum $MnCl_2$ concentrations for the activity of each peak fraction when poly(rA)·$(dT)_{12-18}$ or poly(rCm)·$(dG)_{12-18}$ was used as template-primer. The peak fraction from leukemic spleen was clearly distinguished from the viral enzyme fractions when poly(rCm)·$(dG)_{12-18}$ was used as template-primer. The optimum concentration for the peak from leukemic spleen was the same as that for DNA polymerase γ fraction from spleen of normal rat.

Table 3. Optimal manganese concentrations

Enzymes	Optimum $MnCl_1$ concentrations (mM)	
	Template-primer	
	poly(rCm)·$(dG)_{12-18}$	poly(rA)·$(dT)_{12-18}$
R35 virus	0.2	0.7
MTC PC-I	0.4	0.6
L spleen PC	2	0.7
N spleen PC	2	0.6

For identity of the enzymes see legend to Table 1.
The reaction conditions are specified in Materials and Methods.

Table 4 shows the optimum magnesium acetate concentration for the activity of each enzyme fraction when Mn^{2+} was replaced by Mg^{2+} in the reactions (see Table 3). The peak from leukemic spleen as well as from that of a normal rat required rather a broad range of Mg^{2+} concentrations of 6–15 mM when poly(rA)·$(dT)_{12-18}$ was used as template-primer. However, the viral enzyme fractions gave no detectable activity within any range of Mg^{2+} concentrations tested from zero to 16 mM. When poly(rCm)·$(dG)_{12-18}$ was used as template-primer, none of the four enzyme fractions showed detectable activity with Mg^{2+}.

Table 4. Optimal magnesium concentrations

Enzymes	Optimum magnesium acetate concentration (mM)	
	Template-primer	
	poly(rCm)·$(dG)_{12-18}$	poly(rA)·$(dT)_{12-18}$
R35 virus	n.d.	n.d.
MTC PC-I	n.d.	n.d.
L spleen PC	n.d.	6–15
N spleen PC	n.d.	8–15

n.d. means that the optimum condition could not be determined because essentially no activity was detected.
For identity of the enzymes see legend to Table 1.
The reaction conditions are specified in Materials and Methods.

pH optimum. Table 5 shows that with

Table 5. Optimal pH

Enzymes	Optimum pH	
	Template-primer	
	poly(rCm·$(dG)_{12-18}$	poly(rA·$(dT)_{12-18}$
R35 virus	>9	8.0–8.5
MTC PC-I	>9	8.0–8.5
L spleen PC	>9	7.7–8.0
N spleen PC	>9	7.5–7.8

Potassium phosphate buffer and Tris–HCl buffer were used for pH ranges from 7.0 to 7.5 and from 7.2 to 9.0, respectively.
Other conditions in the reaction are specified in Materials and Methods.
For identity of the enzyme see Table 1.

poly(rA)·$(dT)_{12-18}$ the peak fraction from leukemic spleen as well as from spleen of normal rat required pH values of 7.5–8.0 for optimum activity, while viral enzyme fractions required a little higher pH values of 8.0–8.5. With poly(rCm)·$(dG)_{12-18}$ as template-primer, incorporation of dGMP by the peak as well as by the other three enzyme fractions required pH values of more than 9.

Effect of inhibitor. Poly(rA)·$(dT)_{12-18}$-directed activity of the viral enzymes was inhibited to about 17% by 1 mM *N*-ethylmaleimide. Under the same condition the activity of the phosphocellulose peak fraction from the leukemic spleen or DNA polymerase γ fraction from normal spleen was not inhibited. When poly(rCm)·$(dG)_{12-18}$ replaced poly(rA)·$(dT)_{12-18}$ in the above test, only the viral enzymes were again inhibited. However, 1 mM of *p*-chloromercuriphenyl sulfonic acid inhibited the activity by 90% in all the above cases.

Molecular weight

Phosphocellulose peak fraction from spleen of the leukemic or normal rat was subjected to velocity sedimentation analysis on glycerol gradient. Both enzymes were sedimented at about 4S in solutions containing 0.25 M KCl. The value is equivalent to a mol. wt of 60,000 for a globular protein (data not shown). This value coincided with that reported for rat liver DNA polymerase γ in a high salt condition [26]. However, the relevance to this rather small value remains to be examined. Reverse transcriptase from R35 mammary tumor cells was sedimented also at about 4S. A mol. wt of 70,000 has been reported for R-MuLV [14] and M-MuSV (MuLV) reverse transcriptase [27].

DISCUSSION

The biochemical data obtained in this paper are consistent with the previous knowledge which does not support a viral etiology for tumorigenesis in rats [4, 7]. For example, in the NBU-induced leukemia rat, cell-free transmission was negative; electron microscopic studies and group-specific cell surface antigen were also negative [4].

By phosphocellulose chromatography we showed that the viral DNA polymerase was not noticeably present in the leukemic spleen (Fig. 1a); i.e. the extracts from the leukemic spleen gave only one peak of poly(rA)·$(dT)_{12-18}$-directed activity which coincided with that of DNA polymerase γ. On the other hand, the extracts from R35 virus-producing rat mammary tumor cells gave a unique peak of activity in addition to the peak of cellular DNA polymerase γ. The peak was characterized as the activity of viral enzyme, since it coincided with the activity peak obtained from extract of R35 virions (see Figs. 1c and 1d).

Further characterization on the enzymatic properties of these peaks made clear that the peak corresponding to DNA polymerase γ, either from leukemic spleen, normal spleen, or R35 mammary tumor cells, possessed the same properties, but they were different from those of the unique peak of R35 mammary tumor cells or R35 virions (Tables 2–5). Libertini and Adams [8] observed similar poly(rA)·$(dT)_{12-18}$-directed DNA polymerase activity in carcinogen-induced rat mammary tumor and also in normal lactating rat mammary tissues. They suggested a possibility that the activity is associated with endogenous viruses that may be produced in a normal rat as it is the case in mice. However, in our case, since the phosphocellulose chromatography and tests on enzymatic properties have clearly distinguished the rat viral enzyme from cellular enzyme as described above, we tend to conclude that no viral enzyme was detectable in the rat myelogenous leukemia cells.

It is noteworthy that, in the present study, cellular DNA polymerase γ fractions from rat responded to a synthetic template-primer, poly(rCm)·$(dG)_{12-18}$. This is in contrast to the previous reports claiming that poly(rCm)·$(dG)_{12-18}$ is specific for viral reverse transcriptase [20, 21]. A similar coincidence of the activity directed by poly(rC)·$(dG)_{12-18}$ (in which cytidine residues are not methylated), with that

directed by poly(rA)·$(dT)_{12-18}$, has been reported for DNA polymerase γ isolated from rat mammary tumor and normal mammary tissues [8], HeLa cells [28], or mouse L cells [29]. In our case, however, the activity directed by poly(rCm)·$(dG)_{12-18}$ was lost step by step during further purification of rat DNA polymerase γ by chromatography on a column of DEAE-cellulose of hydroxyapatite (Kozu and Seno, unpublished observations). In the case of mouse myeloma, poly(rCm)·$(dG)_{12-18}$-directed activity is also detectable in a crude DNA polymerase γ preparation but is not detectable in the further purified enzyme preparation (Ono and Matsukage, personal communications). One may therefore be cautious in using this template-primer as a specific means to discriminate viral reverse transcriptase from cellular DNA polymerase, especially when the enzyme preparation is crude. In this respect, further purification and characterization of DNA polymerase γ from rat spleen are under way.

The poly(rA)·$(dT)_{12-18}$-directed activity in the postmitochondrial fractions from the leukemic spleen but not those from normal spleen sedimented at a density of 1.17 g/cm^3 on sucrose (Fig. 2). It seems that the activity is associated with the virions. However, it is noted that the addition of the detergent was not required for the assay of activity, while it was required in the case of R35 virus particles (Fig. 2c). Hence, the activity is presumably of cellular DNA polymerase γ bound to cellular membranes [30] which coincidentally possessed a density value characteristic of RNA tumor viruses. In addition, virus-like particles were not observed in the active fractions by electron microscopy (data not shown). Therefore, the difference observed for postmitochondrial fractions between leukemia and normal spleen may possibly be due to the different growth rate of leukemic cells and normal cells.

Although we failed to detect reverse transcriptase activity in the leukemic cells which had proliferated metastatically in the spleen, this does not necessarily mean that oncogenic virus was not involved in the leukemia induced by NBU in the present study. Sarcomas induced in hamsters or rats with certain types of viruses usually do not reveal the presence of virus particles on electron microscopic examination [1]. In the process of *in vitro* transformation by infection of cells with oncogenic RNA viruses, it is reported that the reverse transcription from viral RNA to DNA occurs in the cells only in the early stages of infection, but it no longer occurs once the transformation is established [31]. By analogy, even if the virus was involved in the rat leukemia in our case, the viral gene may have been active only in a certain early stage of leukemogenesis that occurred in the bone marrow [32] and the viral gene expression could have been suppressed once the leukemic cells were established in the animal. Such possibility could not be ruled out in the present study.

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Effect of Immunotherapy on the Kinetics of Proliferation of a Transplanted Rat Rhabdomyosarcoma

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Abstract—*Our experiment confirms the effect of BCG and of neuraminidase (VCN) associated with mitomycine C (MitoC) treated tumoral cells on a rhabdomyosarcoma serially transplanted in a rat, which have been shown to prevent or to delay the growth of many experimental tumors and also prolonged the survival time of the animals.*

We have determined the effect of active immunotherapy on some kinetic parameters on the tumor growth. The NS 104 tumor may provide a tool to determine, whether this action of immunotherapy on resting cells is also true for other types of tumors.

INTRODUCTION

NON SPECIFIC (i.e. BCG) and/or specific immunostimulations have been shown to prevent or to delay the growth of many experimental tumors.

This effect has been quantified according to various parameters such as: the minimal number of cells which are able to induce in 50% of inoculated animals, the delay of appearance of the tumor, the mean survival time. We report here a study on a rhabdomyosarcoma serially transplanted in rat. We have determined the effects of active immunotherapy of some kinetics parameter of the tumor growth.

MATERIALS AND METHODS

Animals

Six weeks old Fisher 344 rats were obtained from Microbiological Associates, Bethesda, Maryland, U.S.A. and 5 groups of 8 rats were randomly used.

Tumors

NS 104 tumor is a rhabdomyosarcoma induced by subcutaneous injection of 10 mg Nickel Sulphite in Fischer 344 rats and has been provided by The Mason Research Institute, Worcester, Massachusetts, USA.

Bacilli Calmette Guérin (BCG)

Fresh BCG, containing 75 mg of live bacilli has been provided by Pasteur Institute, Paris, France.

Vibrio cholerae neuraminidase (VCN)

Five hundred units/ml of VCN has been provided by General Biochemicals Inc., Chagrin Falls, Ohio, USA.

Mytomycine C (Ametycine)

This product has been provided by Choay Laboratories, Paris, France in dry power.

Experimental protocol

All rats were inoculated subcutaneously on the left flank with 5×10^6 live tumor cells and randomized into 5 experimental groups.

Group I. This group received only live tumor cells.

Group II. Animals of this group were treated with 1 mg of BCG suspended in 0.10 ml RPM 1640 inoculated at the same site as the live tumor cells. The first injection of BCG was done simultaneously with the cells, and a second injection was given 10 days later.

Group III. Animals of this group were inoculated with BCG as in group II, on day 0 and 10 but at a distance of 2 cm from the site of inoculation of live tumor cells.

Group IV. Animals received simultaneously

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live tumor cells and 5×10^7 live tumor cells previously incubated for 1 hr at 37°C with 25 units of neuraminidase (VCN) and 12.50 mg mitomycine C (MitoC). After incubation, cells were washed with fresh RPM 1640 and inoculated subcutaneously in 0.10 ml of medium in the right flank.

A second inoculation of VCN and MitoC treated cells was done on day 10 under the same conditions.

Group V. Animals of this group received 5×10^7 irradiated tumor cells on day 0 and day 10 on the right flank.

Groups I, II and V were repeated in a second experiment. In all groups, rats were palpated daily starting from day 10. Tumors were measured using a calliper. Delay of appearance of palpable tumor were recorded. Four diameters were measured and each one was measured 6 times.

The tumor was measured at least 6 times before death. For each point, the mean diameter was obtained and the volume of the tumor was calculated by assimilation of the tumor shape to a sphere and using the formula $V = \frac{4}{3}\pi(D^3/2)$, V is expressed in cubic centimeters. Results were plotted on a semi-logarithmic paper and the doubling time of the tumor during the exponential growth phase was determined.

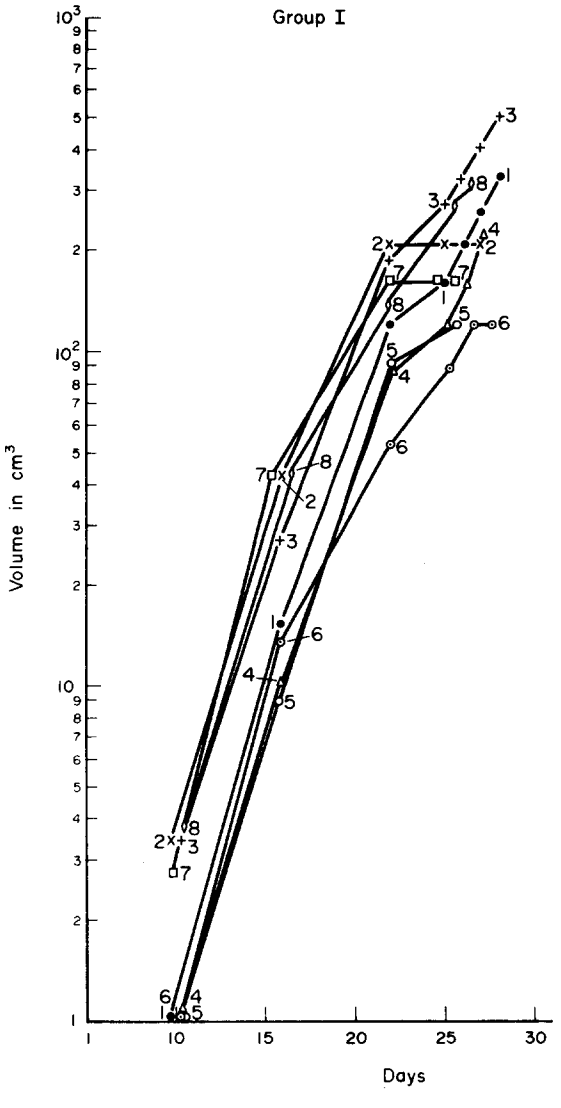

Fig. 1. Eight rats were randomly used in the first experiment of the control group (Group I) and the curves of the Fig. 1 represent the volume growth of the tumours in cm^3 *and the survival time in days for each rat.*

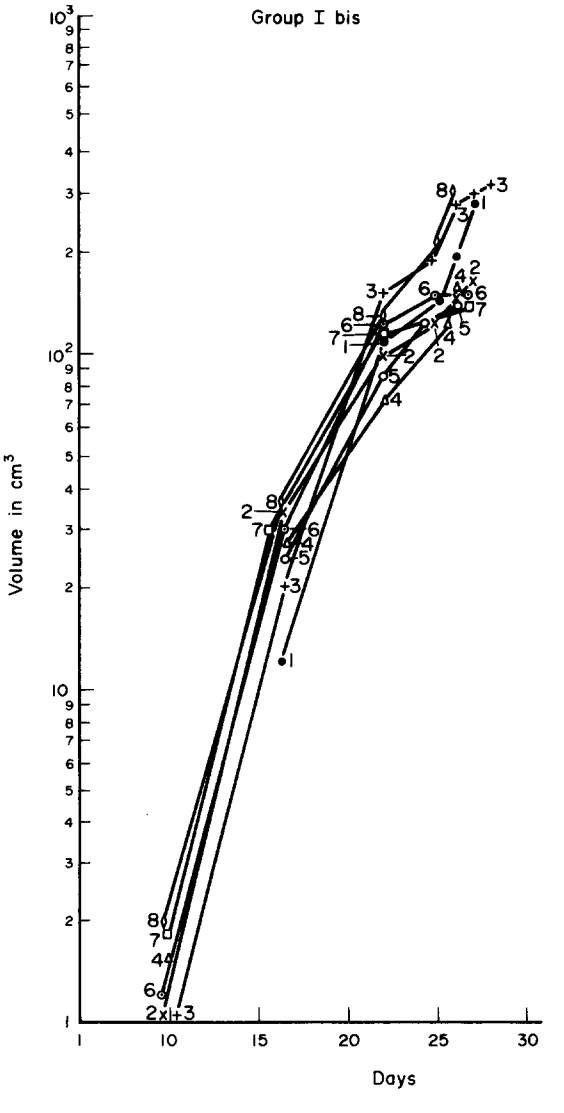

Fig. 2. Eight rats were randomly used in the second experiment of the control group (Group I bis) and the curves of the Fig. 2 represent the volume growth of the tumors in cm^3 *and the survival time in days for each rat.*

For each group, the mean and standard deviation of the doubling time, the survival time and the final volume of the tumor were calculated and compared using a student test.

Correlations between each value, in each group, were made using a computerized programme (compucorps).

Anatomopathological studies

In the first experiment, animals were sacrificed when they were dying.

In the second experiment, 6 animals were used for anatomopathological studies and sacrificed during the plateau phase of the tumor growth. Tumor, spleen, kidney and lymph nodes were systematically taken and fixed with buffered formol and included in paraffine.

Hematein, eosine, safranine, green methyl pyronine and PAS staining techniques were used.

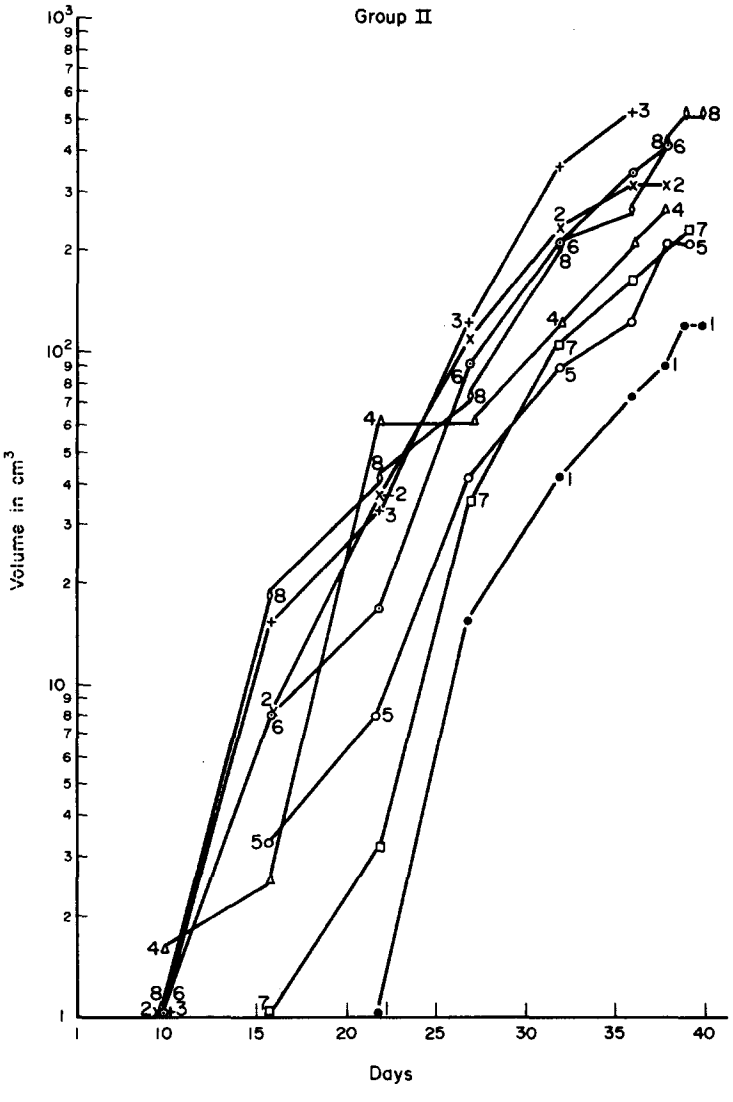

Fig. 3. *Eight rats were randomly used in the first experiment and they were treated with* 1 mg *of BCG suspended in* 0.1 ml *RPM* 1640 *inoculated at the same site as live tumour cells. The first injection of BCG was made simultaneously with cells and a second injection was done* 10 days *later* (*Group* II). *The curves of the Fig.* 3 *represent the volume growth of tumors in* cm^3 *and the survival time in days for each rat.*

RESULTS

Kinetics studies

Figures 1, 3, 5, 6 and 7 represent individual growth curves of tumors from the first experiment, in each group.

Figures 2, 4 and 8: growth curves obtained in the second experiment are represented. The mean values of survival times, doubling

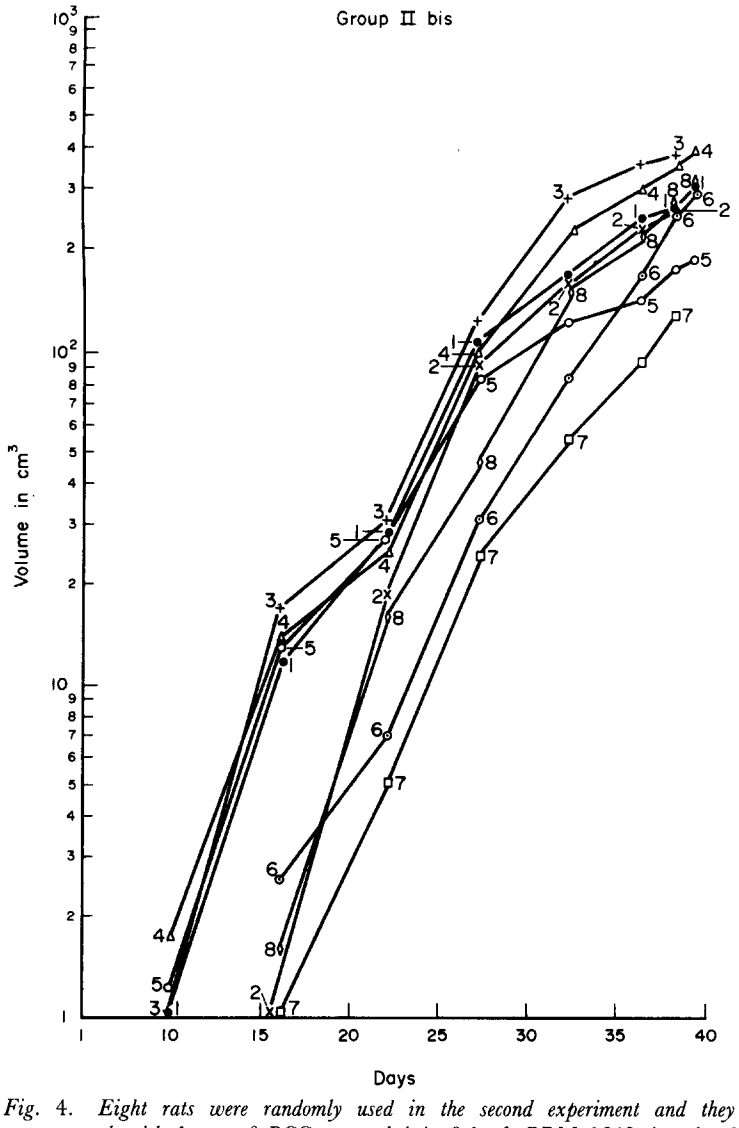

Fig. 4. Eight rats were randomly used in the second experiment and they were treated with 1 mg *of BCG suspended in* 0.1 ml *RPM* 1640 *inoculated at the same site as live tumor cells. The first injection of BCG was made simultaneously with the cells and a second injection was done* 10 days *later (Group* II *bis). The curves of the Fig.* 4 *represent the volume growth of the tumors in* cm^3 *and the survival time in days for each rat.*

times and tumoral volumes are summarized in Table 1.

The mean kinetic results can be summarized as follows:

Tumors from the control group were generally detected on day 10, when they measured 100–300 mm^3. The exponential growth phase of the tumor was clearly defined, mean doubling time of 2.27 ± 0.21 days was calculated. A plateau phase was

Table 1. *Mean values of the survival times, the doubling times and the tumoral volumes of* 5 *groups*

Groups	Survival time of the animals in days	Exponential phase of tumor growth in days		Terminal phase of tumor growth in days		Final volume of tumor in cm^3
		Doubling time	Duration of the exponential phase	Doubling time	Time of the terminal phase	
I	26.75 ± 1.035	2.27 ± 0.21	14.25 ± 3.00	—	—	13.55 ± 6.959
II	38.50 ± 1.309	3.39 ± 0.57	25.60 ± 3.40	—	—	17.36 ± 7.423
III	35.12 ± 0.834	2.72 ± 0.48	19.40 ± 4.70	No growth	3.90 ± 3.50	18.90 ± 5.809
IV	33.50 ± 0.755	3.09 ± 0.47	19.30 ± 2.50	No growth	1.60 ± 0.50	11.26 ± 5.619
V	30.75 ± 4.496	2.68 ± 0.44	17.80 ± 2.50	—	—	13.63 ± 4.314

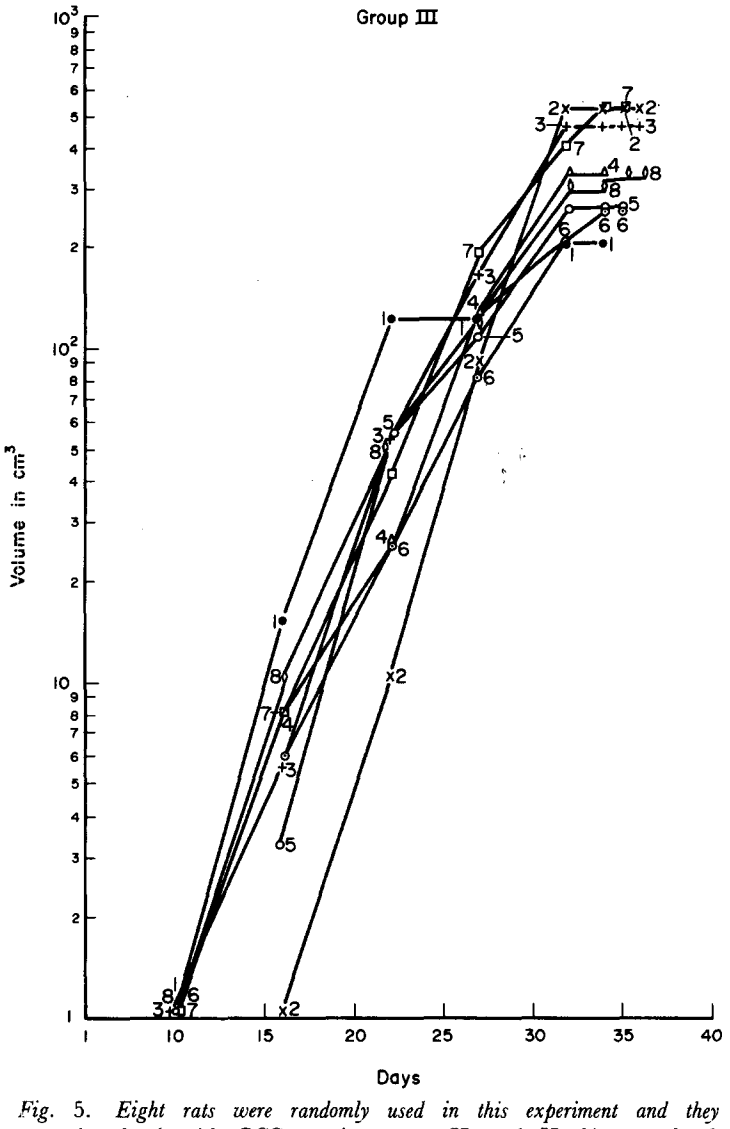

Fig. 5. Eight rats were randomly used in this experiment and they were inoculated with BCG as in groups II *and* II *bis, on* day 0 *and* 10, *but at a distance of* 2 cm *from the site of inoculation of live tumor cells (Group* III*). The curves of Fig.* 5 *represent the volume growth of the tumor in* cm^3 *and the survival time in days for each rat.*

observed only in 2 animals out of 16. The mean final volume of tumor was $13.55 \pm 6.96\,cm^3$.

The inoculation of live BCG mixed with tumor cells on day 0 and 10 produced a clear cut effect on all parameters of tumor growth. In 5 out of 16 animals, tumors were detectable around the 15th day. In all animals, the doubling time of tumors was increased and a mean value of 3.39 ± 0.57 days was calculated. No real plateau phase was observed in this group and tumors reached a volume of $17.36 \pm 7.42\,cm^3$, a value was not statistically different from the control group. The survival time was increased to 38.60 ± 1.31 days.

In group III, the inoculation of BCG at 2 cm from the site of inoculation of tumor cells produced the same effects as the effects of BCG mixed with tumoral cells. The following values were calculated:

The mean value of survival time: $35.12 + 0.83$ days.

The mean value of doubling time: 2.79 ± 0.48 days.

The mean value of tumoral volume: $18.90 \pm 5.81\,cm^3$.

However, in contrast with group II, a clear plateau was observed in 6 out of 8 tumors. The plateau phase probably explains the significant prolongation of survival time which contrasts with a moderate increase of doubling time of tumors.

In group IV, injection of neuraminidase (VCN) associated with mytomycine (MitoC) treated with tumoral cells increased also

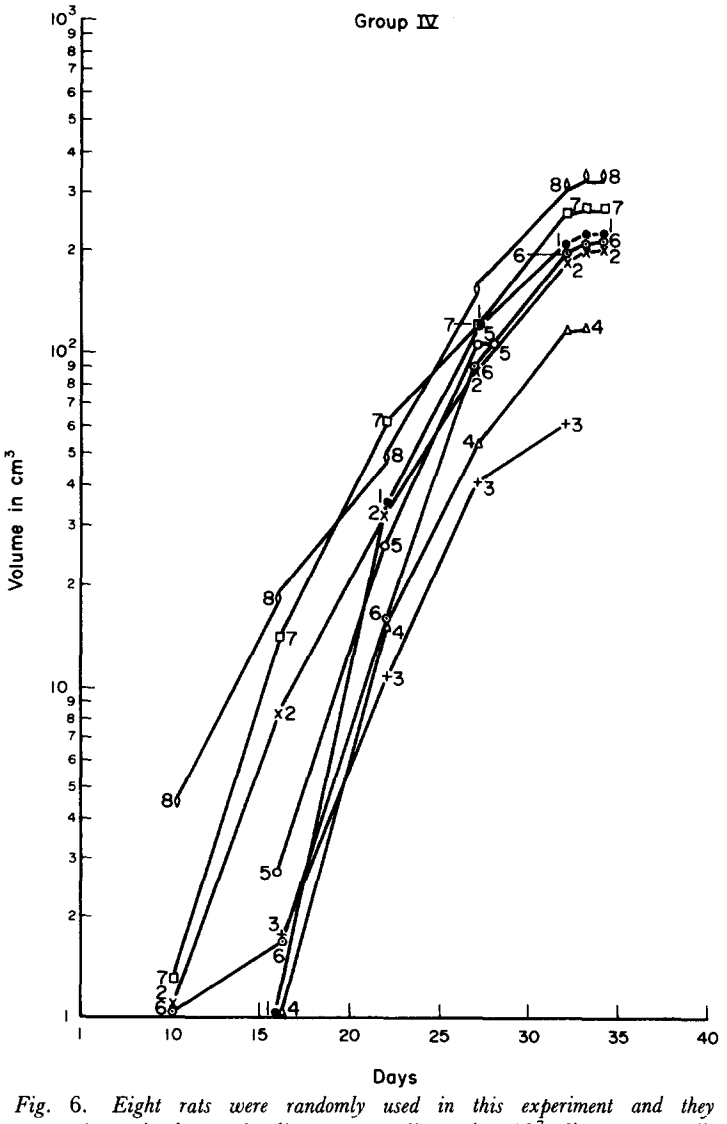

Fig. 6. *Eight rats were randomly used in this experiment and they were given simultaneously live tumor cells and* 5.10^7 *live tumor cells previously incubated for* 1 hr *at* 37°C *with* 25 *units of neuraminidase* (*VCN*) *and* 12.5 mg *of myitomycine C* (*MitoC*) *on* days 0 *and* 10 (*Group* IV). *The curves of Fig.* 6 *represent the volume growth of the tumors in* cm^3 *and the survival time in days for each rat.*

the mean value of survival time: 33.75 ± 0.75 days, the mean value of doubling time: 3.09 ± 0.50 days, the mean value of tumoral volume $11.26 \pm 5.60\,cm^3$. No real plateau phase was observed.

In group V, a great heterogeneity was observed as demonstrated on the curves and by the greater range of standard duration of this group.

Tables 2 and 3 summarize the statistical

Table 2. *Comparison group by group of the doubling time*

	I	II	III	IV	V
I		Different to 0.001	Different to 0.05	Different to 0.001	Different to 0.05
II			Different to 0.05	Not different	Different to 0.02
III				Not different	Not different
IV					Not different
V					

Table 3. *Comparison group by group of the survival time*

	I	II	III	IV	V
I		Different to 0.001	Different to 0.001	Different to 0.001	Different to 0.09
II			Different to 0.001	Different to 0.001	Different to 0.001
III				Different to 0.01	Different to 0.02
IV					Not different
V					

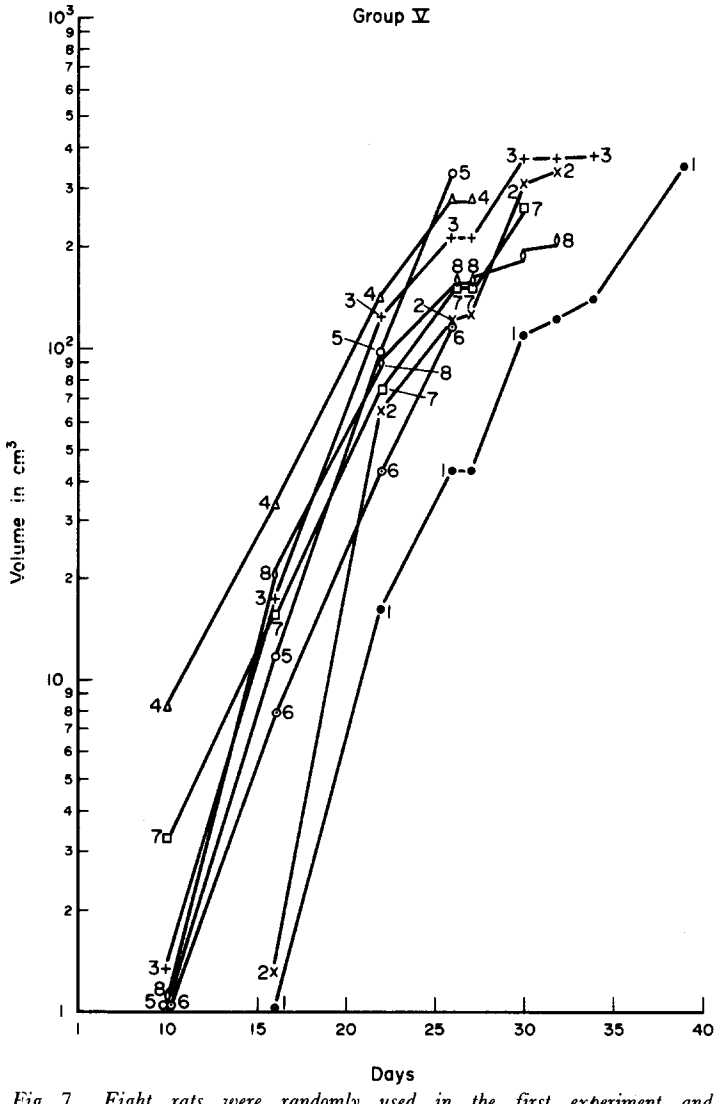

Fig. 7. Eight rats were randomly used in the first experiment and they were given 5.10^7 *irradiated tumor cells on* days 0 *and* 10 *in the right flank (Group* 5*). The curves of Fig.* 7 *represent the volume growth of tumors in* cm^3 *and the survival time in days for each rat.*

comparison of the mean value of survival time and of doubling time of each group.

To determine which parameters were correlated, values from the two experiments were pooled and correlation coefficient were calculated. The results are summarized in Table 4. It appears that the most significant correlation ($r=0.7022$) is observed between the mean value of survival time and of doubling time. The correlation between the mean value of survival time and of tumoral volume is significant at the 1% level ($r=0.4233$). No correlation was found between the mean value of doubling time and tumoral volume ($r=0.1617$, $P<10\%$).

Table 4. Correlations for 70 animals

1.	Correlation of survival time and final volume of the tumor Coefficient of correlation: 0.4233 Significative correlation: $P<1\%$
2.	Correlation of doubling time in the exponential phase of tumor growth and the survival time of the animal Coefficient of correlation: 0.7022 very significant: $P<1\%$
3.	Correlation of doubling time and volume of the tumor Coefficient of correlation: 0.1617 No correlation: $P>10\%$

Anatomopathological studies

The NS 104 tumor has a typical histological aspect of rhabdomyosarcoma [1]. In all groups, large necrotic zones were seen. No peritumoral reaction was seen in the control group. Attempts to quantify this necrosis have been made by measuring its surface. The results are summarized in Table

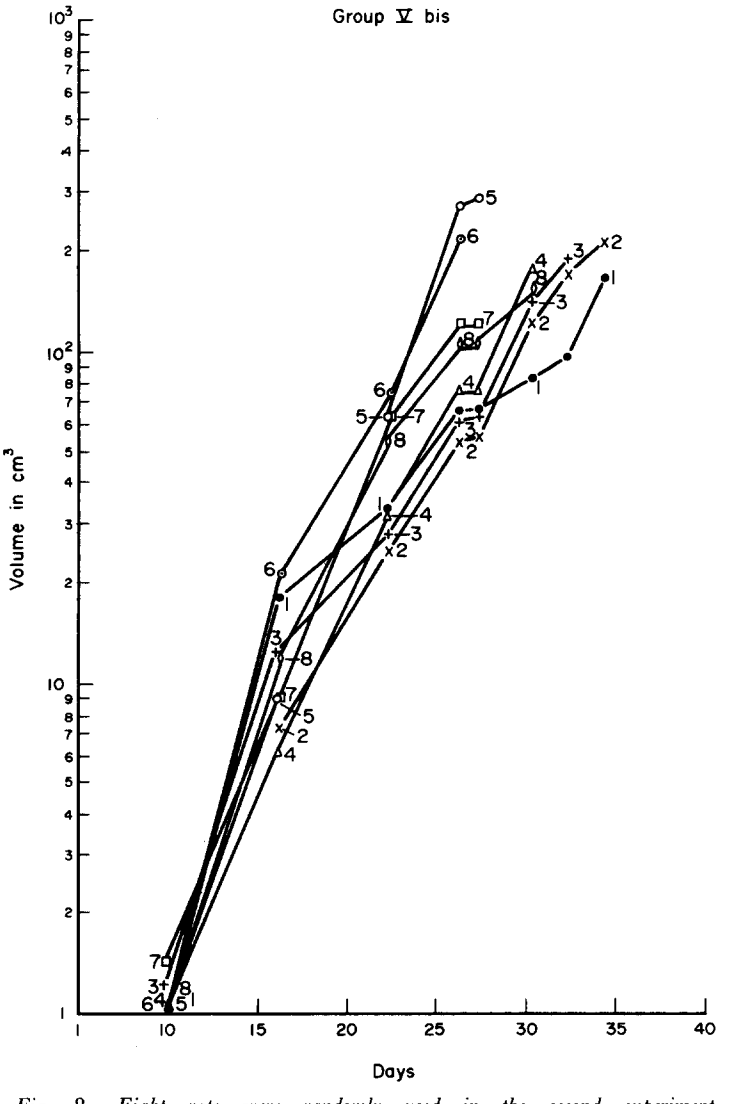

Fig. 8. *Eight rats were randomly used in the second experiment and they were given* 5.10^7 *irradiated tumor cells on* days 0 *and* 10 *in the right flank* (*Group* V *bis*). *The curves of Fig.* 8 *represent the volume growth of tumors in* cm^3 *and the survival time in days for each rat.*

5. The most striking difference was observed in group II, in which necrosis represented around 80% of tumor action. In some sections, the tumor was completely necrotic without living tumor tissues. No significative differences were observed between other groups. It has been noticed, that a liver steatosis was associated with tumor necrosis and the importance of the necrosis was correlated to the extent of tumor necrosis.

Table 5. *Comparative table of the necrosis of the tumor in percentage amongst the* 5 *groups after the histological studies*

No. of the animal	Group I	Group II	Group III	Group IV	Group V
1	37	83	55	26	85
2	34	100	90	30	20
3	32	100	45	35	15
4	43	82	18	30	30
5	21	80	60	60	17
Means	33.33	87.00	52.33	34.33	31.00

Animals of each group have been sacrificed during the last week of their survival time after inoculation of the tumoral cells and the different drugs.

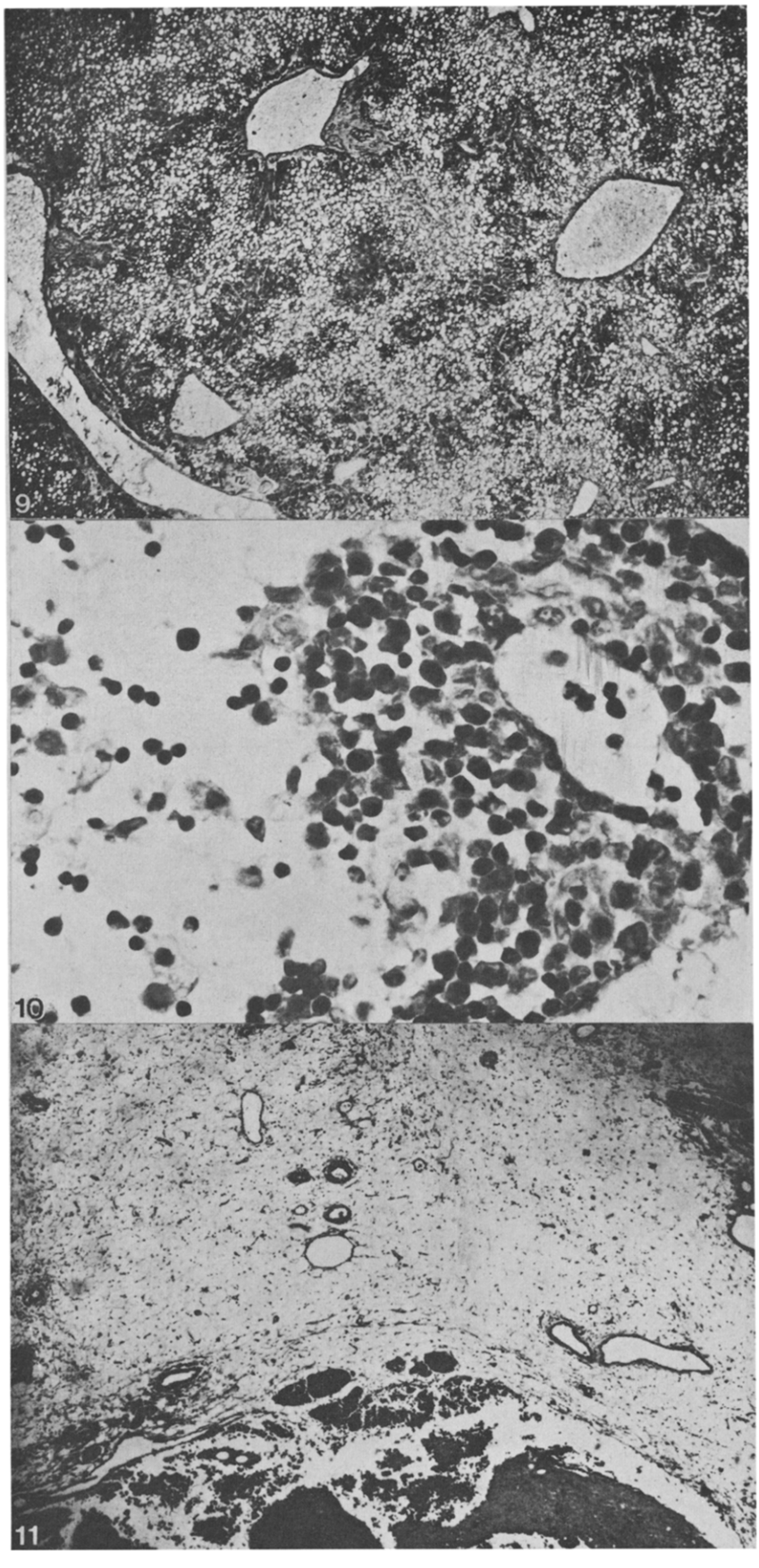

Fig. 9. *Rat liver treated with BCG. There is severe steatosis and considerable vascular congestion, only a few normal cells persist at the border of the portal spaces.*

Fig. 10. *Lymph nodes of the rat treated with BCG shows methyl green pyronine stains many plasma cells next to the sinus.*

Fig. 11. *In this rat treated with neuraminidase (VCN) associated with mitomycine C (MitoC), one observes that the tumor is necrotic but at the margin of the tumor there are only several vessels and some fat tissues without plasmocytic reaction.*

DISCUSSION

Our experiment confirms the effect of BCG and of neuraminidase (VCN) associated with mitomycine C (MitoC) treated tumoral cells on this transplanted rhabdomyosarcoma. The inoculation of BCG mixed with live tumor cells or at 2 cm from the site of inoculation of tumor cells completely prevented the growth of the tumoral cells inoculated in the right flank. BCG was also able to affect the growth of tumor cells inoculated in the opposite flank of the animals. However, all tumor cells multiplied and no animals survived in this experiment.

The different treatments modified significantly the tumoral volume, the doubling time of the tumor and the survival time of the animals.

Both BCG mixed with tumoral cells and to a lesser extent BCG injected near the site of inoculation of tumoral cells were the most effective in prolonging survival time [2, 3].

Treatment with neuraminidase (VCN) [4–6] associated with mitomycine C (MitoC) treated tumoral cells also prolonged the survival time of the animals [7–10]. Injection of irradiated tumoral cells produced a great heterogeneity of response. Some animals died early and were not affected by the treatment whereas other animals had a significant delay of death, indicating that probably the size of the tumor cell inoculum is at the upper limit of efficiency of immunotherapy [11–18].

Measurements of the doubling time indicate that inoculation of BCG mixed with tumor cells affects the most clearly the doubling time of tumor. Comparison of groups III (peritumoral BCG) and IV (Neuraminidase VCN) associated with mitomycine C (MitoC) treated cells shows a slight discrepancy between the survival time and the doubling time. This is explained by the existence of a plateau phase in animals at group III indicating perhaps is some ways a better tolerance to the tumor than in group IV.

Therefore, death might be at least partially related to a toxic effect which may not be directly proportional to the volume of the tumor.

Great variations of the tumor volumes shown by the ranges were observed in each group. This contrasts with the narrow ranges of survival time and doubling time in all but one group.

The absence of correlation between the doubling time and the tumoral volume, and the great correlation between the doubling time and the survival time underlines the importance of determining the doubling time of tumor to detect an anti-tumor effect of immunotherapy on established tumor.

Anatomopathological studies confirms that BCG is able to induce a local and general lymphocytic reaction with a marked peritumoral infiltration and a reaction in draining lymph nodes and in spleen [19–21]. The results of a details study will be reported later.

Our results stress the need for additional studies including the measure of labeled mitosis curves to determine more completely the effect of immunotherapy of kinetic parameters which have not been studied enough in the case of immunotherapy. It has been shown in L1210 leukemia [2, 3] that BCG is acting on cells in the G 1 phase and the availability of other models such as the NS 104 tumor may provide a tool to determine whether this action of immunotherapy on resting cells is also true for other types of tumors.

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Temperature Effects During and After the Diethylnitrosamine Treatment on Liver Tumorigenesis in the Fish, *Oryzias latipes**

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Abstract—*Four experimental groups of the medaka* (Oryzias latipes) *were treated with diethylnitrosamine (DENA) at the concentration of* 100 ppm *for* 8 *weeks. Two of them were kept at a high temperature* (22 ± 2°C) *and others at a low temperature* (5–6°C). *After the DENA treatment, one of the high temperature groups was kept again at a high temperature in normal tap water (H.H-group) and the other was transferred to a low temperature (H.L-group). In the similar way, L.H- and L.L-groups were made. Eight and twelve weeks after the beginning of the experiment, about* 10 *fish in each group were sacrificed for histological observation.*

Both in H.H- and H.L-groups, many tumor nodules were found in their livers. But the number and the size of tumor nodules were larger in H.H-group than in H.L-group. On the contrary, no tumor nodules were observed in L.H- and L.L-groups which were treated with DENA at a low temperature. From these results, an appropriate high temperature is considered to be necessary in the formation and the growth of tumor nodules.

INTRODUCTION

IN RECENT years, several works have been reported about tumorigenesis in aquarium fish by various carcinogens [1–5]. The small aquarium fish, such as zebra danio, guppy and medaka, are thought to be very useful in the research of environmental carcinogens [6], for they are rather susceptible to carcinogens and can be easily reared without special facilities. Besides, fish are available for the experiment to ascertain the temperature effects on tumor induction and tumor growth, because of their poikilothermicity. Recently, we reported that no tumor nodules but only one altered hyperbasophilic focus were induced by the administration of diethylnitrosamine at a low temperature, though almost all the fish treated with DENA at a high temperature had many well developed tumor nodules [7]. In previous experiment the fish had been kept at different temperatures during the DENA treatment. In this work, the process of liver tumorigenesis was divided into two periods, one was the period of DENA treatment and the other was the post-treatment period. Keeping each period at a high or low temperature, the difference in the incidence and the development of the liver tumors in each experimental group were examined and discussed.

MATERIALS AND METHODS

In the present experiment, about 160 fish of himedaka (an orange–red variety of medaka) were used. They were obtained in our laboratory, 9 months old and 23–27 mm in body length. They were kept outdoors before the experiment. In order to be acclimatized to the different temperatures, fish were divided into two lots and kept at 22–25°C or about 5°C for 3 weeks. Then 7 experimental groups were made.

The fish of the 1st and the 2nd groups were administered diethylnitrosamine (DENA, Tokyo Kasei Co., Ltd., Tokyo) in the water at the concentration of 100 ppm for 8 weeks at a high temperature (water temperature = 22 ± 2°C). Then the fish were transferred to normal tap water and kept at a high

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temperature (22±2°C, H.H-group) or at a low temperature (water temperature= 5–6°C, H.L-group) for 4 weeks. The fish of the 3rd group were kept in normal tap water at a high temperature throughout the experiment as a control of the high temperature group (H.C-group).

The 4th and 5th groups were kept at a low temperature (5–6°C) during the DENA treatment. DENA was given at the same concentration for 8 weeks. After the end of the treatment, each of them was transferred to normal tap water and kept for 4 weeks at a high temperature (L.H-group) or at a low temperature (L.L-group). The 6th group kept at a low temperature without DENA treatment served as cold control (L.C-group).

The water was changed once a week throughout the experiment. The fish of the experimental groups were reared in 8 liter glass tanks. They were illuminated with fluorescent light from 6.00 a.m. to 7.00 p.m. The fish were fed with Tetramin (Tetra Werke, West Germany) once a day during the course of the experiment.

When the DENA treatment was stopped and the experiment was finished (8 and 12 weeks after the beginning of the experiment), 8–11 fish in each group were killed and autopsy was carried out. Then the fish were fixed in Bouin's solution. After the samples were transferred into 70% alcohol, the body weight and the liver weight were measured in each individual. The liver-somato indices were calculated. Histological serial sections of whole liver were made by a routine procedure and stained with hematoxylin and eosin.

Besides, as the 7th group, some of the fish treated with DENA at a low temperature were administered DENA successively until 13 weeks under a low temperature condition. After that they were transferred to normal tap water at a high temperature for 4 weeks (L'.H-group). The livers of these fish were also observed histologically.

RESULTS

The results of the liver-somato index are summarized in Fig. 1. Comparing L.C- with H.C-group, it was indicated that the liver weight became heavier at a low temperature than at a high temperature. Electronmicroscopic observation suggested that the increase

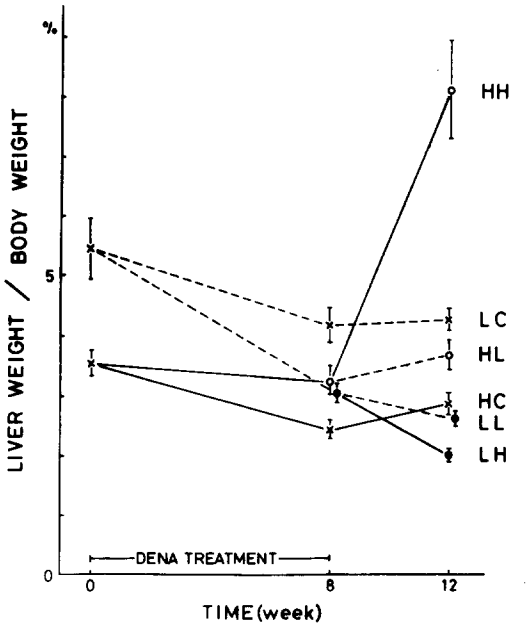

Fig. 1. The ratio of the liver weight to body weight in fish of each group. Experimental procedures were written in the text. The ratio of H.H-group was significantly higher ($P<0.001$) than those of other groups. Broken lines show low temperature period. Vertical bars indicate standard errors.

was mainly owing to the accumulation of glycogen granules in hepatocytes [8].

The fish of H.H-group had markedly enlarged livers. Twelve weeks after the beginning of the experiment, the value was about 8%. It was significantly higher than those of other groups ($P<0.001$). It was clear, by autopsy, that the high value was due to the development of large tumor nodules.

Results obtained from the histological observations of each group are as follows.

Control group (H.C-, L.C-groups)

The livers of the fish fixed at 12 weeks after the start of the experiment were histologically observed. At a high temperature, the fish had reddish livers (H.C-group), but the color became pink when they had been kept at a low temperature for a period of 12 weeks (L.C-group).

The normal liver consisted of polyhedral hepatocytes containing a round to oval nuclei with single centrally-localized nucleolus. The hepatocytes had little stained cytoplasm and showed sheetlike structures along the vascular sinusoides (Fig. 2). Small necrotic parts were occasionally observed in half of the fish kept at a high temperature. However, all fish reared in normal tap water did not show any indication of liver tumorigenesis.

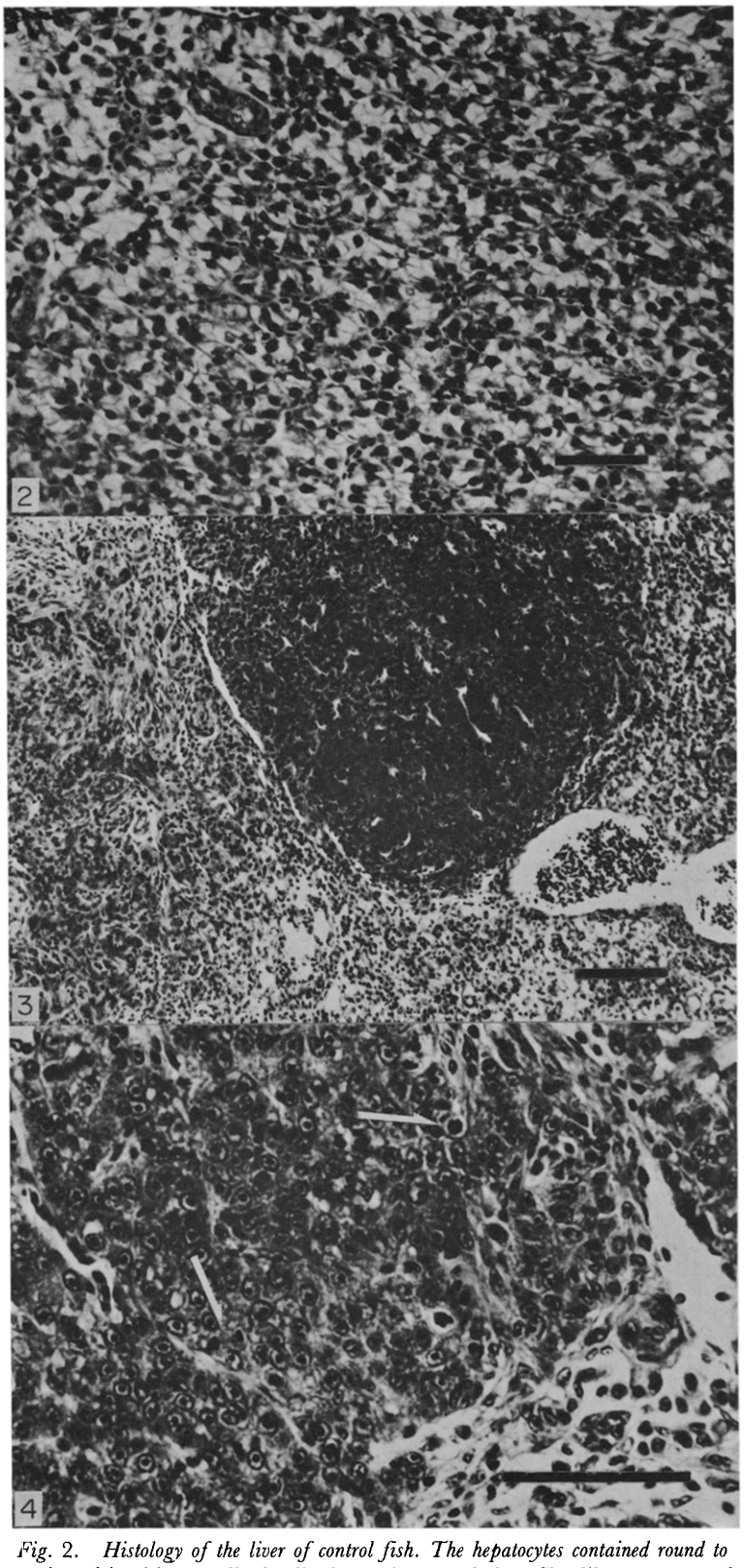

Fig. 2. *Histology of the liver of control fish. The hepatocytes contained round to oval nuclei with centrally localized prominent nucleolus. Sheetlike structures of hepatocytes were observed along the vascular sinusoides. Bottom bar,* 50 μm. *Hematoxylin and eosin (H&E).*

Fig. 3. *Liver of the fish treated with* 100 ppm *of DENA at* 22±2°C *for* 8 *weeks and then kept in normal tap water at the same temperature until* 12 *weeks (H.H-group). Well developed tumor nodule was observed. Bottom bar,* 100 μm. *H&E.*

Fig. 4. *High magnification of tumor nodule of the same fish shown in Fig.* 3. *Mitotic figures were observed frequently. Bottom bar,* 50 μm. *H&E.*

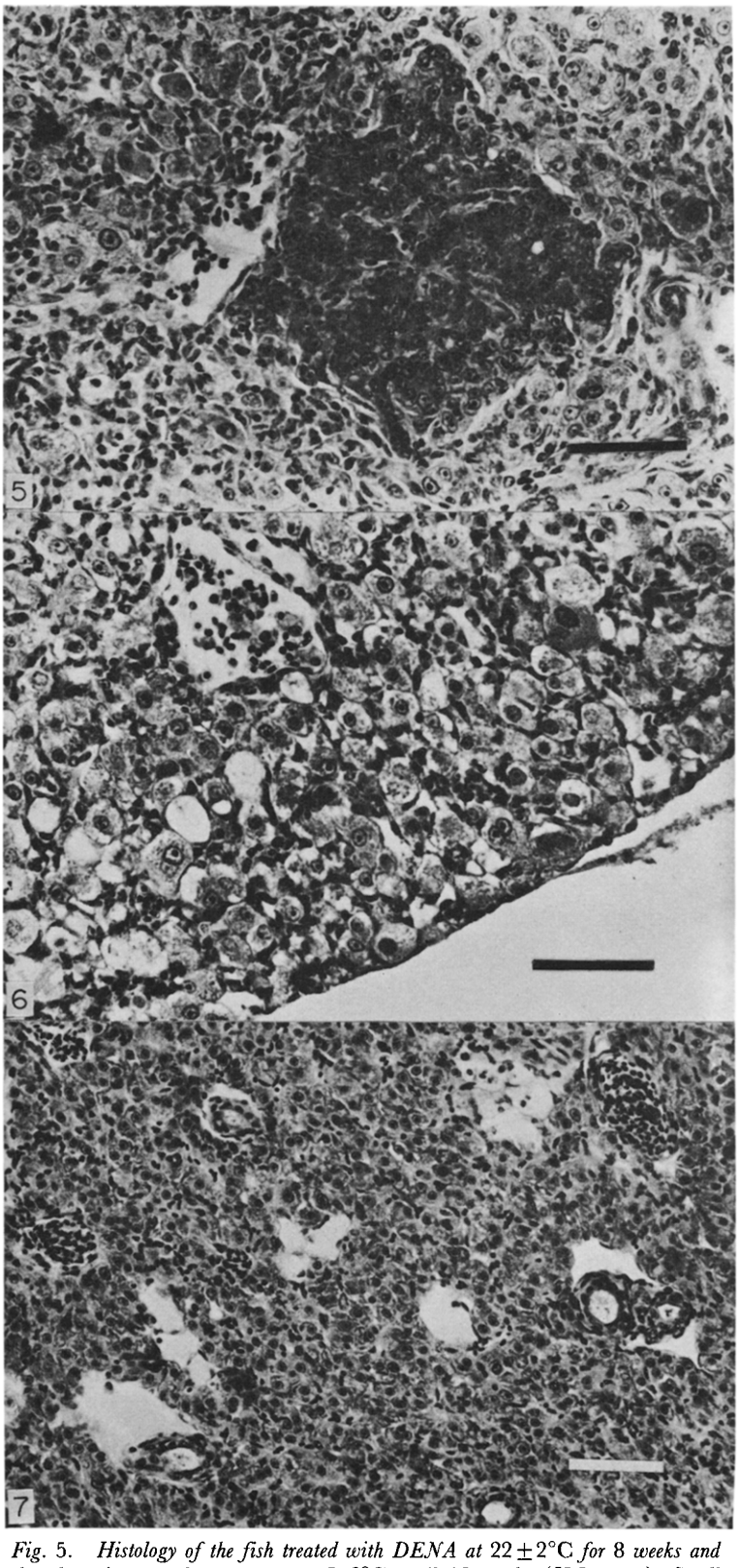

Fig. 5. Histology of the fish treated with DENA at 22±2°C for 8 weeks and then kept in normal tap water at 5–6°C until 12 weeks (H.L-group). Small tumor nodule was observed, but mitotic figure was not found. Bottom bar, 50 μm. H&E.

Fig. 6. Histology of the liver of another fish in H.L-group. Large hepatocytes with eosinophilic cytoplasm were observed frequently in the marginal part of the liver. Bottom bar, 50 μm. H&E.

Fig. 7. Liver of the fish treated with 100 ppm of DENA at 5–6°C for 8 weeks and then kept in normal tap water at 22±2°C until 12 weeks (L.H-group). Some vacant spaces were observed. Hepatocytes contained well stained cytoplasm. Bottom bar, 50 μm. H&E.

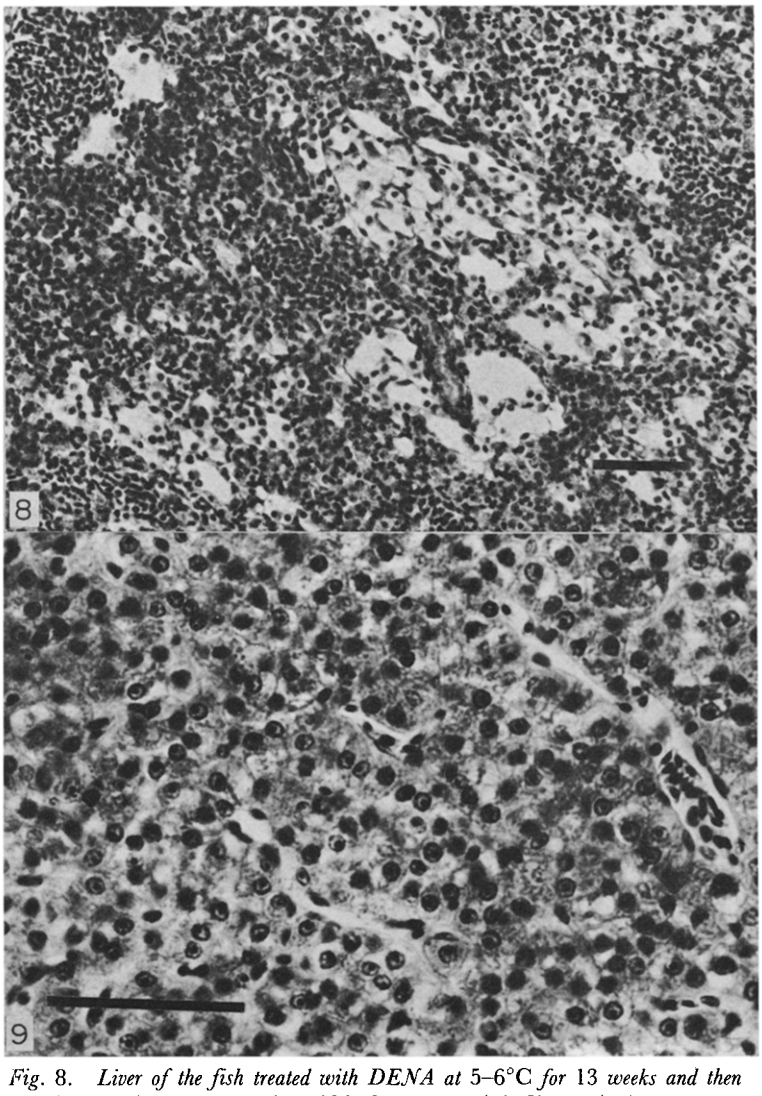

Fig. 8. *Liver of the fish treated with DENA at* 5–6°C *for* 13 *weeks and then kept in normal tap water at* 22±2°C *for* 4 *weeks* (*L'. H-group*). *Many vacant spaces were observed and regular arrangement of hepatocytes found in normal fish was broken down. Bottom bar,* 50 μm. *H&E.*

Fig. 9. *Histology of the liver of the fish treated with DENA at* 5–6°C *for* 8 *weeks and then kept again at the same temperature in normal tap water until* 12 *weeks* (*L.L-group*). *The hepatocytes had round nuclei which frequently contained the nucleolus located on the margin of nucleus. Bottom bar,* 50 μm. *H & E.*

Experimental groups

Eight weeks after the start of the DENA treatment. All the fish treated with DENA at a high temperature showed histologically abnormal livers. They had some small tumor nodules, 1–13 nodules per liver. The size of the tumor nodule was markedly smaller than that of the fish fixed 4 weeks later (H.H-group). Other parts of the liver consisted of enlarged hepatocytes stained eosinophilically and of many non-parenchymal cells.

In contrast to this, all the fish treated with DENA at a low temperature for 8 weeks did not show any histological changes. The livers were similar to those of the control fish kept at a low temperature.

Four weeks after the ending of the DENA treatment H.H-group. All the fish kept at a high temperature during and after the DENA treatment had enlarged whitish livers (11 fish out of 15 fish survived) and some small or large tumor nodules were observed. The tumor nodules seemed to be trabecular hepatoma or liver cell carcinoma. Hyperbasophilic and undifferentiated cells were observed rather frequently in the central part of the nodules. Many mitotic figures were observed especially in small tumor foci (Figs. 3 and 4). These fish had 2–16 tumor nodules per liver, maximum diameter being 100 μm. One fish had so many tumor nodules close together that the number was not able to be counted.

In addition to the hyperbasophilic nodules, hyperbasophilic nodules and the non-parenchymal cells were often observed in the residual parts of the livers. The latter were similar to the cells of connective tissue or lymphatic system.

H.L-group. In this group, the mortality rate was higher than those of other groups after they were tranferred to a low temperature. Eight out of 18 fish survived at the ending. Most of them had whitish livers and also had baso-hyperbasophilic tumor nodules mentioned above. But the nodules were rather smaller (maximum diameter = 28 μm) than those of H.H-group. The fish had 1–7 tumor foci per liver, this number was also smaller than that of H.H-group. It is worth to note that the mitotic figures were scarcely found in these tumor foci (Fig. 5).

In these fish, hyperbasophilic or normal stained altered foci were also observed more frequently than in the fish of H.H-group. Some fish showed the proliferation of bile duct epithelium. One fish did not have any baso-hyperbasophilic foci, but had many abnormal parts containing eosinophilically enlarged hepatocytes (Fig. 6).

L.H-group, L'.H-group. In L.H-group, all of 21 fish survived when they were transferred to high temperature after the DENA treatment. Most of the fish had normal looking reddish livers as those of the control fish. The hepatocytes had well stained cytoplasm compared with the control one. Some small vacant spaces which contained no cells or some cells of connective tissue were observed (Fig. 7). It is highly probable from the observation that some hepatocytes were killed by the DENA treatment.

Four fish out of 5 treated with DENA for 13 weeks at a low temperature (L'.H-group) showed abnormal livers in which mass of hepatocytes were found here and there. The regular structure of hepatocytes was completely broken down (Fig. 8). The liver consisted of many small vacant spaces and of infiltrating non-parenchymal cells.

L.L-group. Thirteen out of 17 fish survived until the end of the experiment. The livers were pink in color and somewhat small in size. No tumor nodules were observed in this group. Some small vacant spaces were also found as well as L.H-group, but the number was not so large in comparison with that of L.H-group. As the histological character, the hepatocyte had a round nucleus which contained the nucleolus frequently localized on the margin of the nucleus (Fig. 9).

DISCUSSION

The results of the present experiment made it sure that the tumor nodules did not appear in the fish treated with DENA at a low temperature. This result was in good agreement with the preliminary experiment. Even though the sufficient time for tumor growth was given at a high temperature after the end of the DENA treatment, no tumor nodules were observed in the fish treated at a low temperature. And only the histological changes were noticed in low temperature groups, which seemed to be induced by the toxicity of the drug as mentioned previously [7].

In the groups treated with DENA at a high temperature, the tumor nodules continued to grow when the fish were kept again at a high temperature after the DENA treatment (H.H-group).

Many mitotic figures were found in these nodules. However, in the fish kept at a low temperature after the DENA treatment, the nodules scarcely grew and the mitotic figures were hardly found (H.L-group). At a low temperature, cell division was inhibited and the tumor nodules were not able to grow. Considering the necessity of an appropriate high temperature in the induction of the tumor nodules, cell division is probably required in the step of the transformation. In fact, it is known that several cell generations are necessary for the fixation and expression of the transformed state in malignant transformation of cultured mouse cells [9].

In mammals, it is well established that nitrosamines become tumorigenic only after the metabolic activation by microsomal enzymes, and that the dealkylated products are related to the carcinogenesis [10–14]. Montesano *et al.* [15] reported that the liver slices of trout and goldfish could metabolize dimethylnitrosamine, but the degree of nucleic acid methylation was very low in comparison with the value of mammals [15]. Although the enzymatic activity and the metabolic pathway of the drug are unknown in medaka, the process of tumorigenesis by DENA is thought to be identical with that of mammals. Ermar could not succeed in tumor induction by the injection of DENA solution in stickleback [16], and the author also failed to observe any histological changes in medaka by a single injection of DENA solution. Since the metabolic activity of microsomal enzyme is very low in fish, chronic accumulation of the damages induced by DENA seemed to be required in the tumor formation. This is thought to be the reason why tumor nodules did not appear by the injection of DENA solution and why an appropriate high temperature was necessary in this experiment. At a high temperature, normal hepatocytes may be killed by the damages induced with DENA and the compensatory cell division during the DENA treatment is probably related to the tumor formation.

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Cytokinetic Changes in Two Cases of Plasma Cell Leukemia Treated with a Multipeptide Derivative of *m*-[di(2-chloroethyl)amino]-L-Phenylalanine (Peptichemio)

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Abstract—*In two patients with plasma cell leukemia (PCL) the cytokinetics of blood and bone marrow plasmocytomatous cells was examined shortly after Peptichemio (PTC 40 mg/alternate days for 6–7 doses) therapy by combined DNA cytofluorometry and* in vitro *tritiated thymidine (^{3}H-TdR) cytoautoradiography. In both cases a rapid fall in circulating plasma cells was induced by the treatment and in one of them the percentage of bone marrow plasma cells was also diminished. After treatment the values of labeling index (LI) and growth fraction (GF) were increased both in blood and bone marrow plasma cells and proliferating bone marrow plasma cells were mostly in early S phase. Their mean grain count (MGC) did not change. Increased population of U-like cells. i.e. damaged cells, was found in the bone marrow. By examining the possible explanations of the findings, the authors relate them to a cellular recruitment following tumor mass reduction. DNA content only was also determined in the blood during the treatment and an increased percentage of* 2n–4n *plasma cells was found. The significance of this last finding is not known at the present.*

INTRODUCTION

THE MITOTIC activity, as expressed by the percentage of tumor cells incorporating tritiated thymidine into DNA (^{3}H-TdR labeling index, LI) is quite low in plasmocytomas at diagnosis [1–8]. In sensitive tumors, alkylating therapy both diminishes the neoplastic cell number and induces a significant increment of the LI among the residual plasmocytomatous cells [1, 6, 8, 9]. This last finding has been regarded by some authors [1, 6, 8, 9] as a proof of the recruitment into the proliferative cycle of previously quiescent cells and, therefore, of an increase of the tumor's growth fraction (GF). This view, however, is questionable, since the simple observation of a raised LI after treatment did not necessarily mean increased proliferative activity [10].

In 1974, we successfully treated 2 patients with plasma cell leukemia (PCL) with Peptichemio (PTC), a multipeptide derivative of *m*-[di(2-chloroethyl)amino]-L-phenylalanine with alkylating and antimetabolic properties [11], and we investigated by combined DNA cytofluorometry and ^{3}H-TdR cytoautoradiography the modifications induced by therapy on other cytokinetic parameters besides the LI. The observed results gave additional information about cytokinetic changes occurring in sensitive plasma cell dyscrasias following tumor mass reduction and are referred to in this paper.

MATERIALS AND METHODS

Two patients with PCL were studied. The first patient (CM) was a 65-yr-old man who was successfully treated for an IgG plasmocytoma, k type, from February

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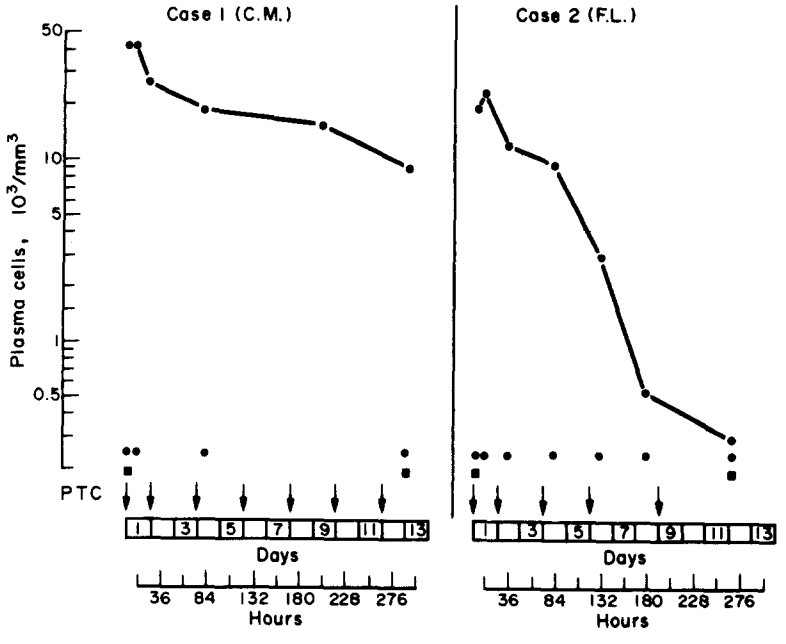

Fig. 1. Dosage and times of PTC administration, times of peripheral blood (●) and bone marrow (■) sampling and fall of circulating plasma cells in two cases of plasma cell leukemia.

to August 1972 with L-phenylalanine mustard (melphalan). Subsequently (October 1972), a short cycle of cyclophosphamide (CTX) was stopped due to pancytopenia. His condition progressively deteriorated up to April 1974 when he was hospitalized in our Institute with the clinical picture of terminal PCL.

The second patient (FL) was a 66-yr-old man who had been suffering from systemic, palpable, small, non-tender lymphnodes since December 1973. He came to our Institute on May 21, 1974. Serum immunoelectrophoresis showed monoclonal IgA paraproteinemia, k type. A lymphnode and bone marrow biopsy disclosed plasma cell infiltrates. The peripheral blood picture was typical of PCL.

The case histories of both patients were given in more detail elsewhere [12].

Both patients were given PTC (Istituto Sieroterapico Milanese, Milan, Italy) as 40 mg infusions during 1 hr, each infusion being repeated at the times shown in Fig. 1.

Peripheral blood (10 ml) and bone marrow (2 ml) samples were collected before, during and after treatment, at the times indicated in Fig. 1.

For each peripheral blood sample, the absolute number of plasma cells per μl was determined by counting 200 nucleated cells on May-Gruenwald-Giesma (MGrG) stained smears. On bone marrow samples, the percentage of plasma cells among nucleated cells (by counting 1000 cells) and their mitotic index (MI) (by counting 3000 plasma cells) were obtained.

On all peripheral blood and bone marrow samples, the nuclear DNA content of 100 randomly selected plasma cells was determined cytofluorometrically on Feulgen stained smears by using a Leitz MPV2 microscope-photometer. The technical characteristics of the instrumentation are detailed by Prenna *et al.* [13] and in our previous papers [14, 15]. The obtained data were evaluated as described elsewhere [14].

Both on blood and bone marrow obtained before and after therapy, the *in vitro* incorporation of ^{3}H-TdR (Radiochemical Amersham Center, S.A. 1.9 Ci/mM) was evaluated according to Foadi *et al.* [16]. The incubation time was 1 hr. The autoradiographs were prepared using Kodak NTB2 nuclear track emulsion and then exposed at 4°C for one week. The labeling index (LI) was determined by counting 4000 plasma cells and the mean grain count (MGG) by examining 50 labeled cells. The growth fraction (GF) was evaluated on the same autoradiographs according to the method of Gavosto *et al.* [17] recently confirmed by Matera and Masera [18].

On bone marrow autoradiographs only, the nuclear DNA content of ^{3}H-TdR labeled and unlabeled plasma cells was determined according to Ernst and Killmann [19].

Table 1. Mitotic index, percent frequency of the classes of DNA values, labeling index, growth fraction and mean grain count in a case (No. 1, CM) of plasma cell leukemia during and after chemotherapy

Tissue	Hr*	Plasma cells†	MI (%)	Classes of DNA values 2n	2n–4n	4n	LI (%)	GF (%)	(MgC±DS)
Peripheral blood	0	42.2	—	94	5	1	0.25	9.1	(82±20)
	8	41.8	—	81	19	0	—	—	—
	80	19.0	—	81	19	0	—	—	—
	288‡	9.1	—	81	19	0	0.50	16.3	(81±21)
Bone marrow	0	94	6.3	84	12	4	0.77	22.4	(85±25)
	288‡	91	1.3	52	38	10	2.11	49.8	(88±30)

*After the start of chemotherapy;
†Absolute number/μl in the peripheral blood and percent of bone marrow nucleated cells;
‡24 hr after the last dose of PTC.

Table 2. Mitotic index, percent frequency of the classes of DNA values, labeling index, growth fraction and mean grain count in a case (No. 2, FL) of plasma cell leukemia during and after chemotherapy

Tissue	Hr*	Plasma cells†	MI (%)	Classes of DNA values 2n	2n–4n	4n	LI (%)	GF (%)	(MGC±DS)
Peripheral blood	0	19.0	—	92	7	1	0.70	3.2	(91±16)
	8	24.0	—	79	21	0	—	—	—
	32	12.0	—	82	18	0	—	—	—
	80	9.5	—	84	16	0	—	—	—
	128	3.0	—	87	13	0	—	—	—
	176	0.5	—	89	11	0	—	—	—
	264‡	0.3	—	94	6	0	0.98	5.1	(87±20)
Bone marrow	0	60	5.3	87	11	2	2.78	20.7	(90±18)
	264‡	40	1.3	82	14	4	3.20	30.7	(81±25)

*After the start of chemotherapy;
†Absolute number/μl in the peripheral blood and percent of bone marrow nucleated cells;
‡72 hr after the last dose of PTC.

RESULTS

Figure 1 shows: (1) the doses and the times of PTC administration, (2) the times of blood and bone marrow sampling and (3) the fall of the circulating plasma cell number during treatment in the two cases of PCL studied.

The cytologic, cytofluorometric and cytoautoradiographic findings are reported in Tables 1 and 2 and in Figs. 1–3.

Case 1 (CM)

In the first patient, during the period of study, an exponential decrease of circulating plasma cells was observed (Fig. 1), while the percentage of bone marrow plasma cells was not significantly reduced (Table 1).

Concomitantly with their numerical fall, the DNA content distribution of circulating plasma cells was modified. The percentage of diploid (2n) cells was reduced (from 94 to 81%), while the population of cells with 2n–4n DNA content (2n–4n plasma cells) was increased (from 5 to 19%). Twenty-four hours after the last PTC administration, the ^{3}H-TdR LI of the same cells was twice that observed before therapy (Table 1).

In bone marrow plasma cells, 24 hr after the last dose of PTC, both the MI and the percentage of diploid cells were distinctly lower than before therapy, while the cells with 2n–4n and 4n DNA content, as well as the ^{3}H-TdR LI and GF, were increased (Table 1).

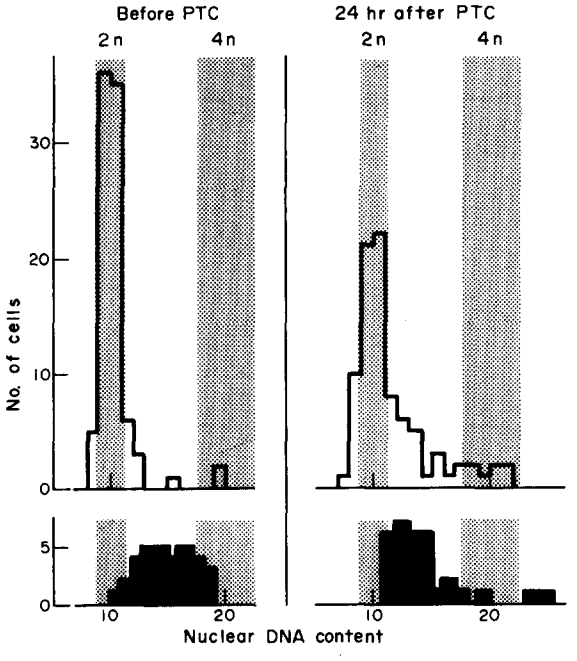

Fig. 2. Distribution of the nuclear DNA content of ^{3}H-*TdR unlabeled* (□) *and labeled* (■) *bone marrow plasma cells in the case* 1 (*CM*).

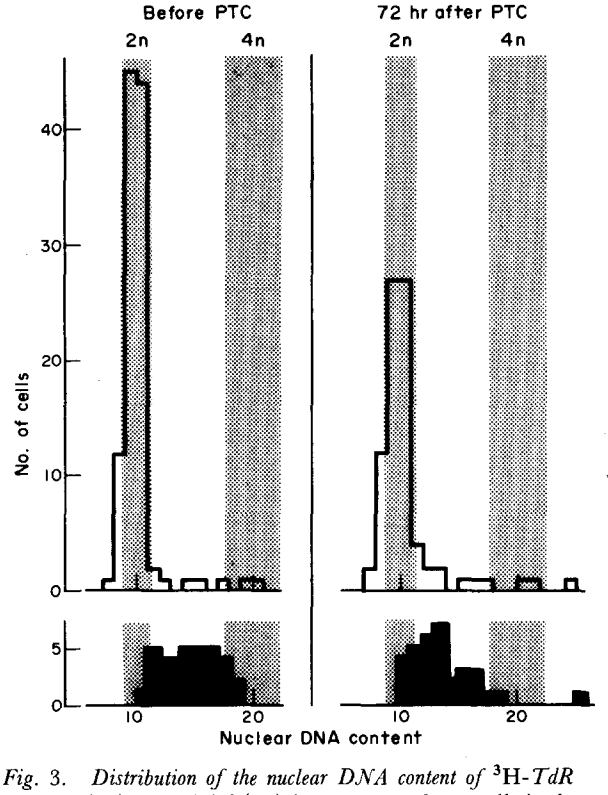

Fig. 3. Distribution of the nuclear DNA content of ^{3}H-*TdR unabeled* (□) *and labeled* (■) *bone marrow plasma cells in the case* 2 (*FL*).

The rate of ^{3}H-TdR incorporation, as expressed by the MGC, was not significantly different before and after therapy, both in the blood and bone marrow proliferating plasma cells (Table 1).

In bone marrow before therapy, the DNA values of ^{3}H-TdR labeled plasma cells were fairly evenly distributed between the 2n and 4n modes (Fig. 2), while 24 hr after therapy they were more frequent (78%) in the early S phase. On the contrary, before therapy the great majority of ^{3}H-TdR unlabeled plasma cells was diploid and some (10%) had 2n–4n DNA content (U-like cells). After therapy this last aliquot was much higher (27%).

Case 2 (*FL*)

In the second patient, the circulating plasma cell decrease during therapy was more conspicuous and rapid than in the first one (Fig. 1), and the percentage of bone marrow plasma cells was also diminished (Table 2).

The cytokinetic changes during and after PTC therapy were substantially similar to those observed in the first case.

Minor differences can be summarized as follows: (1) in the peripheral blood the percentage of plasma cells with 2n–4n DNA content increased during the first 32 hr of the treatment (Table 2) but, unlike in the first patient (Table 1), returned to the pretreatment value thereafter; (2) in the bone marrow, 72 hr after the last dose of PTC, the increase of the population of both 2n–4n plasma cells (Table 2) and of the U-like cells (Fig. 3) was less pronounced than in the first case (Table 1 and Fig. 2) 24 hr after PTC; (3) at the above times after therapy, the LI and GF of both blood and bone marrow plasma cells were less increased in the second (Table 2) than in the first patient (Table 1).

DISCUSSION

In our patients with PCL, successful PTC therapy has altered plasma cell kinetics in a similar way, both in bone marrow and in peripheral blood. We will consider separately the bone marrow and peripheral blood findings. Differences between the two cases in relation to quantitative variation of some cytokinetic parameters will be discussed later.

In bone marrow the main cytokinetic changes after PTC can be stated as follows: (a) increase of ^{3}H-TdR labeling index (LI) and of growth fraction (GF); (b) decrease of the population of 2n and increase of the percentage of 2n–4n plasma cells; (c)

build up of DNA-synthesizing plasma cells in early S phase and (d) increased frequency of U-like plasma cells.

An augmented ^{3}H-TdR LI in bone marrow plasma cells has been already reported [1, 8, 9] in plasmocytomas after alkylating-induced tumor mass regression. As formerly stated in acute leukemia [20, 21, 22], a recruitment into cell cycle of previously quiescent cells following tumor mass reduction has been postulated by Salmon [8] in such cases. This view, although accepted by Alberts and Golde [9] and by Drewinko *et al.* [1], has been recently questioned by Baccarani and Santucci [10], since the finding of a raised LI alone after treatment is not adequate proof of increased tumor GF. Cell recruitment has been strengthened however by Pileri *et al.* [6, 23] from cyto-autoradiographic evidence of activated macro-molecular metabolism, i.e. increased RNA and protein synthesis and actinomycin binding, accompanying LI and GF rise in plasmocytomatous cells a few days after CTX therapy.

In this context, change of the DNA distribution pattern concomitant with LI and GF increases in plasmocytomatous cells observed in our patients shortly (24–72 hr) after PTC treatment, further substantiate Salmon's hypothesis [8].

Indeed, although the increased frequency of 2n–4n cells (Table 1 and 2) cannot be ascribed to increased mitotic activity only, since U-like cells are also present (Figs. 2 and 3), the prevalent distribution of DNA synthesizing bone marrow plasma cells in early S phase strongly suggests that several diploid cells have entered into proliferation at the same time, i.e. are recruited.

Furthermore, cell recruitment is also supported by examining other alternative explanations of the LI increase.

The augmented frequency of U-like plasma cells after treatment (Figs. 2 and 3) is in accordance with the known greater effectiveness of PTC [11, 24], as are other alkylating agents [25–27], on proliferating rather than on quiescent cells. Therefore this finding opposes the possibility of preferential cell kill of diploid cells.

The occurrence of DNA repair in non-lethally damaged plasma cells, already excluded in plasmocytomas treated with melphalan [9], is not likely, since its demonstration requires a cytoautoradiographic exposure time longer than the seven days employed by us. Moreover, DNA repair is evidenced by a nuclear grain count much lower than that observed in our cytoautoradiographs [28, 29].

Finally, a delayed cell progression through S phase, as observed in acute leukemia blasts after 1-β-D-arabinofuranosylcytosine (ARA-C) [20, 21, 30] or amethopterin (MTX) [20, 31], can account for increased LI. A lowered grain count over labeled nuclei is reported, however, in such an event [30, 32], but is not observed after PTC in our cases (Tables 1 and 2).

In light of the above findings, cell recruitment is the most likely explanation of the cytokinetic changes that occurred after effective PTC treatment in PCL. The decrease of MI is not contrary to this interpretation, since it is probably dependent on a delay in G_2 progression [33], as evidenced by the augmented frequency of tetraploid plasma cells (Tables 1 and 2).

The LI increase of bone marrow plasma cells was quite modest in our patients, as compared to those reported in patients treated by other authors [1, 6, 8, 9].

Differences in planning the investigations can account for this finding. In fact we studied cytokinetic changes a short time (24–72 hr) after repetitive PTC schedule, whereas the above authors studied them 3 weeks after melphalan–prednisone therapy [1, 9] or a few days after high doses of CTX administered for 3 consecutive days [6]. This can imply that, in our patients, a more conspicous LI increase was hampered by the fact that the repeated PTC administrations killed the newly proliferating, i.e. recruited cells, as suggested by the augmented frequency of U-like cells after the end of therapy.

As far as the peripheral blood findings are concerned, the changes of LI, GF and DNA distribution of plasmocytomatous cells *after therapy* were similar, although less pronounced, to those observed in the bone marrow (Tables 1 and 2); their significance is probably the same as that of the bone marrow changes. On circulating plasma cells only, we further studied the DNA distribution *during therapy* and observed, in both cases, an increase of the population of 2n–4n cells (Table 1 and 2), as already reported by us [34] and by Lutz *et al.* [35] in peripheral blasts from acute leukemias during combination therapy. Since in ours and in Lutz's studies cytoautoradiography was not combined, it was not clear whether 2n–4n cells

were recruited or U-like or S-synchronized cells. In subsequent studies we were able to examine the peripheral blood of leukemic patients during ARA-C or vincristine (VCR) treatment by both DNA flow cytofluorometry and ^{3}H-TdR cytoautoradiography [36]: increased aliquots of 2n–4n cells were also found which were mostly U-like cells after VCR, whereas an increased percentage of labeled cells was also observed after ARA-C. The significance of 2n–4n circulating cells after PTC must be ascertained by similar methodological approach.

As already mentioned, we must now consider why the magnitude of some cytokinetic changes, i.e. of the increase of the aliquot of 2n–4n plasma cells and of their LI and GF, has been greater after therapy in the first (CM) than in the second (FL) patient (Tables 1 and 2).

Since the 2n–4n plasma cells were mostly U-like cells (Figs. 2 and 3), their more pronounced increase in the first patient can be accounted for by the fact that bone marrow and blood samples were obtained in this case sooner (24 hr after the last administration of PTC) than in the second (72 hr after the last administration of PTC), since the U-like cells are rapidly cleared by macrophages.

The greater LI increase in the first patient must be related to the fact that in this case PCL was the terminal event of a plasmocytoma, whereas in the second PCL was a primitive disease. As pointed out by Bergsagel and Pruzanski [37], the plasmocytoma doubling time is greatly shortened during the "acute terminal phase" and this can account for the more rapid and marked cell recruitment that occurred in case 1.

The origin of recruited cells is debated at present.

Pileri *et al.* [6, 38] have proposed that circulating B-lymphocytes, most of them carrying and synthesizing in culture membrane Ig idiotipically identical to the M component [39–42], or morphologically unrecognizable stem cell, as in mouse plasmocytoma [43], account for both tumor supply in steady state and recruitment after chemotherapy. According to these authors, such functions cannot be accomplished either from the proliferating plasma cell compartment, since it is not a stem compartment but only a transit one toward differentiation [5], or by the non-proliferating plasmocytomatous cells, owing their high differentiation in Ig synthesis and the stability of their macromolecular template [44, 45].

The problem however is still unsolved.

Salmon has recently grown clusters of neoplastic plasma cells in agar cultures [46].

Moreover, in immunized rats lymphnodes, DNA synthesis has been observed in plasma cells producing IG and/or specific antibodies [47, 48]. Such findings show that plasmocytic differentiation does not hamper proliferative activity. Whether or not steady tumor supply and recruitment after therapy originate from B-lymphocytes or from plasma cells, or from both, is yet to be demonstrated.

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Leukocyte Migration Inhibitory Activity in the Serum of Patients Affected by Hodgkin's Disease and Other Immunoproliferative Diseases

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Abstract—*Leukocyte migration inhibition was studied by employing the agarose plate method. The inhibitory effect of blood sera from healthy subjects, from patients affected by lymphomas or other immunoproliferative diseases and from patients with diseases not involving the immuno-system has been investigated.*

No inhibitory effect was detected in the sera of patients affected by various pathology including four solid neoplasia.

It was possible to demonstrate the presence of an inhibitory factor in the sera of patients affected by Hodgkin's disease as in sera of patients affected by other immunoproliferative diseases.

INTRODUCTION

THE LEUKOCYTE migration inhibition test (LMIT) [1] has been widely employed for the evaluation of cell-mediated immunity in human malignant neoplasia [2–7]. This method represents a useful means to elucidate the problem of the existence of serum "blocking" factors which interfere with the antitumoral activity of lymphocytes and macrophages [8–12]. In fact if the presence of such "blocking" factors can be definitely proved, then the progression of the tumor mass, in spite of an immunological anti-tumor reactivity, can be explained.

Recently these serum factors have been studied by the LMIT in a considerable number of patients with malignant neoplasia [13]. The results of these studies increased the interest for this research. However, employing the agarose plate method [14–18] which is more rapid than the capillary system and has a good reproducibility [19], we studied the effect of blood sera of healthy subjects, of patients with lymphomas or other immunoproliferative disease and with diseases not involving the immuno-system. The migrating leukocytes were obtained from healthy donors.

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MATERIALS AND METHODS

Patients

The study was carried out on 18 healthy normal donors and on 40 patients. They were divided in three groups: Hodgkin's disease (HD), immunoproliferative disease as defined by Dameshek [20, 21] and miscellaneous disease. All the patients were untreated. The diagnosis was based on clinical and haematological findings and in HD on histological examination of lymph nodes and spleen.

Sera

Five millilitres of blood were collected from each patient and donor. The blood was centrifuged at $800 \times g$ for 10 min, then the sera to be tested were stored at 4°C for not less than one week.

Preparation of leukocytes

Blood from 25 healthy donors was especially employed to prepare the leukocytes used for migration. No more than three sera were tested against each leukocyte suspension.

Twenty millilitres of heparinized blood from fasting donor were diluted 3:1 v/v with Emagel (Behringwerke) and were allowed to sediment for about 45 min at 37°C in inverted plastic syringe. The supernatant was taken, centrifuged at $800 \times g$

for 10 min and the cellular sediment (leukocytes and erythrocytes) was washed three times at 800 × *g* for 10 min.

A final cell suspension of 25–30 × 10^6 leukocytes was prepared.

Hanks' solution without Ca^{2+} and Mg^{2+} (HS) (Eurobio) supplemented by 20 mM *N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES) (Eurobio) was employed both for washing and final cell suspension.

Agarose gel preparation

A solution containing 73.3% of a pure agarose (Behringwerke) 0.94% solution in distilled water previously prepared at 100°C, 12.5% of fetal calf serum (Difco) and 14.2% of HS with HEPES, was prepared in a water-bath at 50°C.

Migration inhibition assay

Two millilitres of agarose gel, prepared as previously described, were poured into Petri dishes (35 mm ϕ, Falcon Plastic). After the gel had solidified, holes (ϕ 2.5 mm) were punched and 5 μl of leukocyte suspension were introduced into each one. The test sera were put into the holes at final concentration of 16 or 32%. In the reference holes the sera were replaced by HS with HEPES. All the assays were carried out in quadruplicate. The dishes were incubated for 21 hr at 37°C in humid atmosphere.

The diameters of migration areas were measured and the leukocyte migration index (LMI) calculated as:

$$\frac{\text{average diameter test}}{\text{average diameter reference}} \times 100.$$

Statistical methods

Mean and standard deviation of the mean were calculated by standard methods.

Differences between means were calculated according to the *t*-test for unpaired samples. A difference was considered significant when $P < 0.1$ or less.

The migration index was considered significant when it was smaller than 89 and 87% respectively for 16 and 32% concentrations of sera.

RESULTS

Normal sera did not produce a considerable migration inhibitory effect either at 16% or at 32% and in both cases the S.D. from the mean was low (Table 1).

The LMI of 14 sera from patients with miscellaneous diseases showed a mean value of 94.09 ± 4.70 at 16% and of 92.28 ± 4.99 at 32% without significant variation from the normal mean. In fact significantly lowered values found only in 3 patients (16%) at 16% and in 2 patients (14%) at 32%. (Table 2).

The mean LMI in 17 patients with HD was 83 ± 15.5 at 16% and 70.1 ± 19.2 at 32%. These values are significantly lower

Table 1. *Leukocyte migration index of the sera from healthy donors*

Patient	Age	Sex	WBC/mm^3	Lymphocytes %	LMI Serum 16%	LMI Serum 32%
1	23	M	4800	38	94.0	93.0
2	36	M	5600	39	95.0	91.0
3	27	F	4700	42	93.0	92.0
4	31	M	5300	44	93.2	95.0
5	33	M	6400	42	92.5	94.5
6	29	F	4600	39	93.5	91.5
7	40	M	6500	45	95.0	92.0
8	35	M	6800	38	95.0	94.5
9	22	F	5200	43	92.5	92.0
10	26	M	7000	44	93.0	92.5
11	39	M	6200	42	100.0	99.0
12	44	F	5300	48	98.2	97.7
13	26	M	7200	41	99.1	98.0
14	29	M	6300	48	100.0	99.0
15	30	F	5700	45	97.5	98.5
16	34	M	6000	44	98.5	99.0
17	26	F	4800	38	99.1	99.5
18	32	M	6200	44	97.0	99.0
				M.V. ± S.D.	95.8 ± 2.6	95.4 ± 3.1

Table 2. Leukocyte migration index of the sera from patients affected by miscellaneous diseases

Patient	Age	Sex	Diagnosis	WBC/mm³	Lymphocytes %	LMI Serum 16%	LMI Serum 32%
19	50	M	Chronic myelocytic leukemia	128,000	3	88.2	89.5
20	52	M	Chronic myelocytic leukemia	32,000	8	96.0	88.0
21	40	M	Chronic active hepatitis	3000	32	94.0	94.0
22	65	M	Peptic ulcer	5000	37	95.0	95.0
23	24	M	Hypoplasic kidney	8400	29	95.0	95.0
24	69	M	Rheumatoid arthritis	6700	35	98.2	97.7
25	47	M	Thromboangiitis obliterans	5300	38	97.0	95.0
26	81	M	Gastric carcinoma	7600	32	99.0	97.2
27	63	M	Chronic pancreatitis	6300	27	83.0	82.0
28	48	M	Gastric carcinoma	6200	41	95.0	88.6
29	41	M	Intestinal carcinoma	5800	45	91.5	93.0
30	43	F	Rheumatoid arthritis	7200	19	88.7	84.2
31	61	M	Carcinoma of the prostate	8400	29	97.5	96.7
32	18	M	Diabetes mellitus	5600	35	99.2	96.0
					M.V. ± S.D.	94.09 ± 4.70	92.28 ± 4.99

Table 3. Leukocyte migration index of the sera from patients affected by Hodgkin's disease

Patient	Age	Sex	WBC/mm³	Lymphocytes %	LMI Serum 16%	LMI Serum 32%
33	70	M	5800	32	86.0	30.0
34	69	M	20,600	8	94.0	86.7
35	45	M	5700	28	94.0	62.0
36	29	F	5600	22	93.0	94.0
37	16	M	4800	27	75.5	73.5
38	61	M	4000	32	92.0	83.0
39	17	M	8100	31	88.8	88.8
40	36	F	8700	30	27.7	27.7
41	37	M	6000	39	81.9	77.1
42	20	M	3400	28	82.3	76.4
43	76	F	5000	40	94.4	77.7
44	39	M	4500	20	74.2	44.1
45	70	M	7200	38	94.2	76.4
46	73	F	3500	29	87.1	76.4
47	65	M	28,500	12	89.0	87.2
48	22	F	12,000	16	72.1	55.5
49	61	M	3200	28	86.1	75.1
				M.V. ± S.D.	83.0 ± 15.5	70.1 ± 19.2

Table 4. Leukocyte migration index of the sera from patients affected by immunoproliferative diseases

Patient	Age	Sex	Diagnosis	WBC/mm³	Lymphocytes %	LMI Serum 16%	LMI Serum 32%
50	39	M	Multiple myeloma	7400	35	95.0	93.0
51	54	M	Multiple myeloma	6000	32	98.0	96.7
52	69	M	Multiple myeloma	7600	43	73.2	70.5
53	66	M	Chronic lymphocytic leukemia	94,500	94	88.1	47.0
54	62	M	Chronic lymphocytic leukemia	28,300	83	93.0	41.1
55	67	F	Chronic lymphocytic leukemia	151,500	98	94.1	96.4
56	23	M	Acute lymphoblastic leukemia	90,000	84	88.7	86.7
57	40	M	Non-Hodgkin's lymphoma	18,000	23	46.5	29.4
58	56	M	Non-Hodgkin's lymphoma	20,000	48	81.4	70.5
					M.V. ± S.D.	84.22 ± 16.08	70.23 ± 25.46

than normal ($P<0.001$ both at 16 and 32%). It should be noted that the patients with significantly lowered LMI were 11 (65%) at 16% and 14 (82.5%) at 32%. (Table 3).

The sera of 9 patients with immunoproliferative diseases showed a mean LMI of 84.22 ± 16.08 at 16% and of 70.23 ± 25.46 at 32% which are values significantly lower than normal ($P<0.01$ at 16% and $P<0.001$ at 32%). It is important to point out that cases Nos. 53, 54 and 57 (two cases of Chronic Lymphatic Leukemia and one case of non Hodgkin's lymphoma) had a particularly low LMI, especially at 32%. Moreover we can observe that the patients with significantly lowered LMI were 5 (55%) at 16% and 6 (66%) at 32% (Table 4). All these results are summarized in Fig. 1.

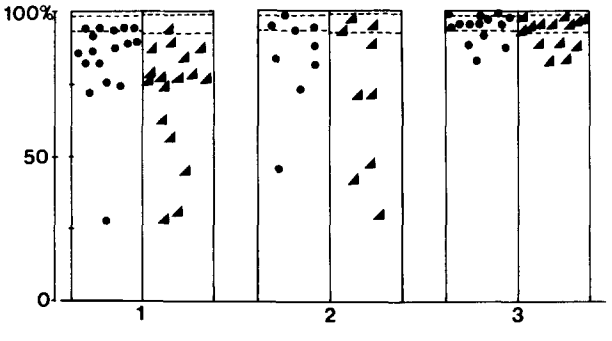

Fig. 1. Leukocyte migration index of the sera from patients affected by Hodgkin's disease (1) Immunoproliferative diseases (2) Miscelleeaneous diseases (3).

The area within the two dotted lines represents the normal mean value ± S.D. of 18 healthy donors.

● *Serum concentration* 16%.

▲ *Serum concentration* 32%.

DISCUSSION

The sera of healthy donors did not cause inhibition of leukocyte migration and the statistical analysis shows the good reproducibility of the method we used.

In the sera from patients with Hodgkin's disease we were able to demonstrate a factor capable of inhibiting leukocyte migration (LMIF) even at the lowest concentration used in our experimental protocol.

This factor was present even in the more heterogeneous group of immunoproliferative diseases, but it should be pointed out that the two cases of CLL and the single case of non-Hodgkin's lymphoma greatly contribute to lowering the mean value, especially at higher serum concentration.

No inhibition of leukocyte migration was detected in the sera of our patients which we refer to using the term of "miscellaneous diseases"; moreover it should be noted that four sera from solid neoplasia were included in this group.

Finally, comparing our results with other authors studies [13] it is important to point out that we did not observe the increase in leukocyte migration found in the sera of some patients with malignant neoplasia.

As yet we have insufficient information to decide whether to ascribe the factor to tumoral antigen–antibody complexes present in the sera in cases of solid neoplasia [22–26] or to the macroglobulinemic factor found in sera of patients affected by Burkitt's lymphoma [27] or whether it may be connected with the lymphocytotoxic factors found in sera from patients affected by Hodgkin's disease or by autoimmune disease [28–37].

We must remember that recently this factor was found also in infectious diseases [38] and by the same authors it was correlated to a good delayed hypersensitivity of the patient [39]. This last finding correlates well with the "MIF-like" nature of the serum factor detected in some patients with lymphoproliferative diseases [40].

Our work confirms the interest of the method of Clausen in evaluating this property of sera from patients with cancer and in observing that LMIF seems particularly related to the proliferative diseases of the immune system.

Our group is now involved in further studies on the nature of this factor.

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The LMI Assay in Lung and Colorectal Cancer: The Use of Allogeneic Extracts Prepared by Different Methods

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Abstract—*We have examined the effects of simple tumour homogenates, and of* 3MKCl *and papain extracts upon the* in vitro *migration of leucocytes obtained from* 56 *patients with lung or colo-rectal cancer and from* 43 *control individuals.*

Although there was considerable overlap between the ranges of results, leucocyte migration was significantly slowed in response to the crude homogenates, in response to two of the three 3MKCl *colorectal tumour extracts and to the* 3MKCl *lung tumour extract, as compared with results obtained from patients without cancer, or in patients whose cancer was of the alternative type. No responses, tumour specific or otherwise, were obtained when a papain extract was used and when a third* 3MKCl *colorectal extract obtained by an identical method to the two other extracts was used.*

INTRODUCTION

THE LEUCOCYTE migration inhibition (LMI) test is an *in vitro* assay that correlates well with delayed hypersensitivity *in vivo* [1]. It has been used extensively to demonstrate specific tumour-directed immunological reactivity of lymphocytes from patients with cancer [2–9]. We have observed a greater degree of leucocyte migration inhibition with a crude colorectal tumour extract in patients with large bowel cancer than in matched non-cancer controls [2], but the marked overlap between the two groups made interpretation of individual results difficult. By using a more refined tumour extract, it might be possible to improve the discriminative value of the LMI test. Tumours can be solubilized by 3M potassium chloride and by papain digestion, and extracts prepared in this way have been shown, by lymphocytotoxicity assays [10], by lymphocyte transformation and by cutaneous delayed hypersensitivity reactions [11] to contain tumour specific antigens. We have therefore repeated the LMI test in further groups of patients with colorectal and with lung cancer using such extracts, in an attempt to separate the responses of the cancer patients from those of the non-cancer patients more clearly. A secondary virtue of the test lies in its relative simplicity, which allows the test to be used in demonstrating the ability of a tumour extract to elicit tumour-orientated responses and thus justifying the investigation of such active extracts in other *in vitro* techniques.

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MATERIALS AND METHODS

Patients

Blood samples were obtained from 30 patients awaiting surgery for carcinoma of the colon or rectum, 26 pre-operative patients with primary lung carcinoma and 43 hospital in-patients, who were suffering from a variety of non-malignant conditions such as vascular disease, peptic ulcer and diverticular disease. All patients appeared to be in reasonably good health. None had received blood transfusions or immunosuppressive therapy. The mean ages of the colorectal cancer patients, the lung cancer patients and the non-cancer controls were 62.3, 62.0 and 64.3 yr respectively.

Leucocyte preparation

Leucocytes were harvested from heparinised

peripheral venous blood after separation by simple sedimentation for 1 hr at 37°C.

The cells were then washed thrice in Eagles Minimum Essential Medium (MEM) and then resuspended in the requisite volume of Eagles MEM supplemented with 10% foetal calf serum.

Preparation of extracts

(i) 3MKCl *extracts.* Individual tumour specimens were obtained within 30 min of excision and then stored at −20°C. Three 3MKCl colon tumour extracts (I, II and III) were prepared, each one from pools of 18-tumour specimens, as follows. The tumour specimens were thawed, dissected free from fat, normal and necrotic tissue and minced with scissors. Fifty grams of the pooled tumours were resuspended in 200 ml 3MKCl and homogenized using an Ultraturrax homogenizer operated at $\frac{1}{3}$ to $\frac{1}{2}$ maximum output. The homogenate was incubated for 18 hr at 4°C with stirring. The suspension was centrifuged at 78,000 × ***g*** for 60 min and the soluble supernatant dialysed against H_2O, concentrated by dialysis against aquacide, and dialysed against phosphate buffered saline, pH 7.3, overnight. Finally the preparation was centrifuged at 78,000 × for 60 min and the soluble fraction collected and stored at −20°C. A lung tumour extract was also prepared by an exactly similar method.

(ii) *Papain extract.* This was prepared from a single patient who died with metastatic colon carcinoma. The tumour tissue was taken from hepatic metastases 8 hours post mortem. After dissecting it free from normal liver and necrotic debris, the tissue was minced with scissors, and suspended in 0.25M sucrose, 1 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6 at a concentration of 4 ml/g wet weight prior to homogenization. The preparation of the soluble "antigen" fraction was performed by limited papain digestion of a crude membrane fraction of a tumour homogenate as described by Baldwin *et al.* [10]. Soluble proteins, obtained by elution of the papain digest from a DEAE-cellulose ion exchange column using 0.5 M Tris–PO_4, at pH 7.8, were precipitated by the slow addition of saturated ammonium sulphate to the extract to give a final concentration of 66.7%. The precipitated proteins were sedimented at 600 × ***g*** for 15 min and redissolved in phosphate buffered saline (pH 7.3) (PBS). The resulting solution was dialysed at 4°C for 24 hr against three changes of PBS. This fraction was taken as the final antigen preparation and has been shown specifically to inhibit lymphocytotoxicity of colorectal cancer patients' cells for cultured colorectal tumour cells [10].

(iii) *Tumour homogenates.* Minced tumour tissue was suspended in PBS at 4 ml per gm wet weight of tissue and then homogenized using an Ultraturrax at approximately $\frac{1}{3}$–$\frac{1}{2}$ maximum output until 80 to 90% cellular disruption had been achieved. Suspensions were centrifuged at 1500 × ***g*** for 30 min and the supernatants were collected and stored at −20°C.

All procedures in the preparation of extracts were performed at 0–5°C unless otherwise stated. Protein concentrations were determined using the method of Lowry *et al.* [12] with crystalline bovine serum albumin as the standard.

LMI assay

For this assay we used our previous technique [2], and also the modification described below. Briefly, a series of plastic tubes were prepared by the addition to each of 100 μl of tumour extract at the required concentration. One hundred microlitres of a final leucocyte suspension were then added to each of these tubes. The contents of the tubes were mixed by gentle agitation and then allowed to incubate for 1 hr at 37°C in a humidified 5% CO_2 in air atmosphere. Control cultures consisted of leucocytes with 100 μl PBS in place of tumour extract. After incubation the leucocytes were resuspended and then drawn up into triplicate 25 μl glass capillary tubes (Drummond Microcaps), one end of each then being sealed with 'Cristaseal', inert clay (Hawksley glass). The capillary tubes were then centrifuged for 150 × ***g*** for 5 min, collecting the cells at the sealed end. Each capillary was then cut at the cell-medium interface and the cell containing portions mounted in the wells of plastic migration plates (Sterilin Ltd.), embedding the sealed ends in a little siliconized stopcock grease. The wells were filled with medium and a circular glass coverslip carefully applied, avoiding air bubbles. The wells of the migration plates were then coded and the plates incubated for 16 hr at 37°C in a humidified 5% CO_2 in air atmosphere. After incubation the plates were removed, the fan of leucocytes in each migration well projected and traced onto paper, and the areas of migration measured by planimetry.

Several concentrations of each extract were examined in the assay. The concentrations used ranged between 0.5 and 4 mg/ml because it was found that the optimal concentrations varied for each of the active extracts. The number of tests performed on each patient's blood sample was determined by the leucocyte yield.

Calculations

The mean areas of migration for each set of triplicate capillaries were calculated, and then a Migration Index (MI) derived by the formula:

$$\text{Migration Index} = \frac{\text{Mean Area of leucocyte migration with extract}}{\text{Mean Area of leucocyte migration without extract}}$$

The results obtained with the leucocytes from patients from each diagnostic group were compared by the Wilcoxon rank sum test to assess the significance of differences of migration. All *P*-values are 2-tailed.

RESULTS

Table 1 gives the results obtained with the crude homogenate and papain extracts, whilst Table 2 gives the results obtained with extracts obtained by the 3MKCl method.

Crude homogenates of both lung and colorectal cancers significantly inhibited white cell responses of patients with the same tumour type, but not with the other tumour type ($P<0.01$ for the comparison of responses to colorectal cancer extract by colorectal and lung cancer patients' white cells and $P<0.01$ for the comparison of responses to lung cancer extract by lung cancer and colorectal cancer patients' white cells (Wilcoxon rank sum test).

No significant responses or trends could be obtained in response to the papain extract in the patients tested.

Again, no difference in migratory responses were noted between cells obtained from colorectal cancer patients or hospital non-cancer controls when 3MKCl extract I was used. However, changes were noted in response to extracts II and III and to the lung cancer extract. Responses to colorectal cancer extract II differed significantly in colorectal cancer patients' white cells and in those of lung cancer and non-cancer controls ($P<0.02$ and $P<0.01$ respectively, Wilcoxon test). Likewise there were significant differences between the responses of colorectal cancer patients' white

Table 1. *Leucocyte migration inhibition responses to papain extracts and homogenates of tumours in controls and in lung and colorectal cancer patients*

Extract	Mean Migration Index	No. of patients tested	S.E.M.	Significance of differences
Papain colorectal cancer				
Patients				
Non-cancer control	0.96	7	0.03	$P>0.1$
Colorectal cancer	1.01	6	0.01	
Colorectal cancer homogenate				
Patients				
Non-cancer control	1.08	6	0.02	$P<0.01$
Colorectal cancer	0.87	6	0.04	
Lung cancer	1.12	8	0.05	$P<0.01$
Lung cancer homogenate				
Patients				
Non-cancer control	1.04	9	0.03	$P<0.02$
Lung cancer	0.88	7	0.05	
Colorectal cancer	1.08	7	0.02	$P<0.01$

Table 2. Leucocyte migration inhibition responses to 3 different 3MKCl extracts of colorectal cancer and to a 3MKCl lung cancer extract in controls and in lung and colorectal cancer patients

Extract	Mean Migration Index	No. of patients tested	S.E.M.	Significance of differences
Colorectal Cancer I				
Patients				
Non-cancer control	1.01	7	0.02	$P>0.1$
Colorectal cancer	0.98	9	0.02	
Colorectal Cancer II				
Patients				
Non-cancer control	1.16	8	0.04	$P<0.01$
Colorectal cancer	1.03	9	0.02	
Lung cancer	1.21	8	0.06	$P<0.02$
Colorectal cancer III				
Patients				
Non-cancer control	1.04	9	0.02	$P<0.02$
Colorectal cancer	0.88	8	0.05	
Lung cancer	1.08	7	0.03	$P<0.01$
Lung cancer				
Patients				
Non-cancer control	1.08	8	0.04	$P<0.05$
Lung cancer	0.91	12	0.05	
Colorectal cancer	1.06	7	0.03	$P<0.05$

cells to extract III as compared with responses in lung cancer and non-cancer controls, ($P<0.01$, $P<0.02$ respectively, Wilcoxon test). Similarly, the 3MKCl lung cancer extract significantly inhibited the responses of lung cancer patients' white cells as compared with those of colorectal cancer or control patients ($P<0.05$, $P<0.05$ respectively, Wilcoxon test). Responses to colorectal cancer extract II manifested themselves as an apparent loss of a stimulatory response seen when a colorectal cancer patients' white cells were incubated with a colorectal cancer extract, whereas with extract III the responses showed as apparent inhibition of migration in response to a similar tumour extract.

There was considerable overlap between the responses of control and test groups, of patients' cells and this is illustrated in Figs. 1 and 2 which show the results from all the tests performed with the tumour homogenates and with the 3MKCl colorectal extract III and 3MKCl lung cancer extract respectively. These two figures indicate that half of the responses of patients with lung cancer or with colorectal cancer fell within the normal range as defined by mean ± 2 S.D.

DISCUSSION

Cell-mediated immune reactions to tumour extracts have been demonstrated in a variety of human cancers by both *in vitro* and *in vivo* methods. The extraction procedure used in preparing the antigen may be of critical importance to the success or failure of any particular assay system. Aqueous extracts have been used with some success to produce specific inhibition of both leucocyte migration and adherence amongst patients with, for example, colonic, breast and lung cancers, and malignant melanoma [3–5, 13–15]. Our results likewise show the specific activity of simple extracts. The value

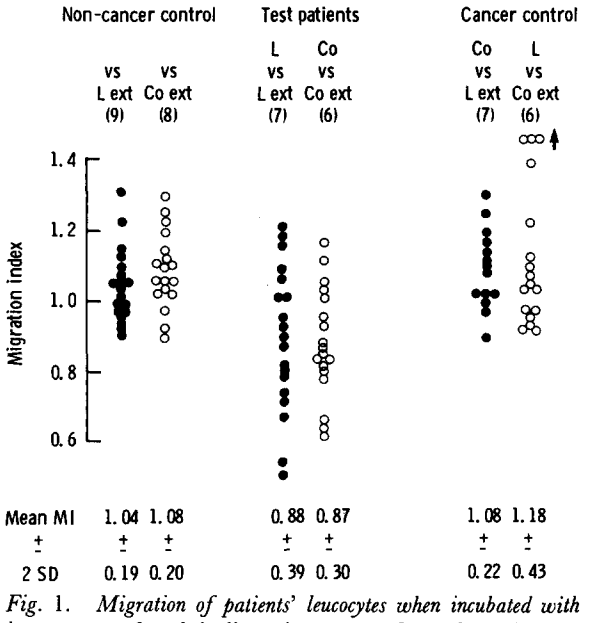

Fig. 1. Migration of patients' leucocytes when incubated with homogenates of pooled allogeneic tumours. Co = colorectal cancer patients. L = lung cancer patients. Co. Ext. = extract of colorectal cancer. L. Ext. = extract of primary lung cancer.

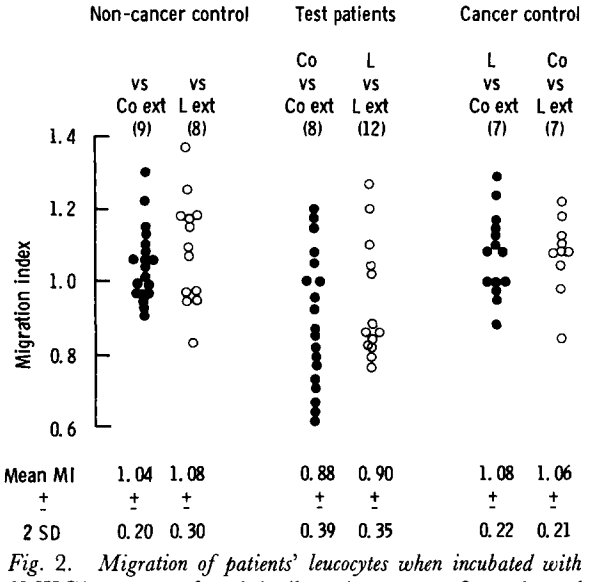

Fig. 2. Migration of patients' leucocytes when incubated with 3MKCl *extracts of pooled allogeneic tumours. Co = colorectal cancer patients. L = lung cancer patients. Co. Ext. =* 3MKCl *colorectal cancer extract* III. *L. Ext. =* 3MKCl *lung cancer extract.*

of papain extracts (which contain tumour cell membrane proteins and glycoproteins) has not been fully assessed. Papain-solubilized material prepared from leukaemic blood has been shown, by serological methods, to contain a leukaemia specific antigen [16] and a colon tumour extract prepared in this way can specifically inhibit lymphocyte cytotoxicity for human colon cancer target cells maintained *in vitro* [10], but using this same extract we were unable to demonstrate tumour specific reactivity in the LMI test. Two possible reasons for this are failure to choose the correct extract concentration and failure to remove blocking material from the leucocyte preparations, but both these are unlikely. The extract concentrations we used covered a wide range extending to levels which include those used in showing migration inhibition in the presence of non-papain solubilized extracts. The importance of repeated washing of leucocytes has been emphasized [17] and we adopted a standard washing procedure which has proved effective in the past [2] and in using the other extracts in this study. Unfortunately, there was insufficient extract to test a larger number of patients in the LMI assay. In view of the relatively low yield of extract when using the papain digestion method [10], we then concentrated on the other extraction methods. Nevertheless, we feel that papain extraction is probably unsuitable for preparing antigen for use in the LMI test, though more extensive testing is clearly needed.

3MKCl extracts contain a broad spectrum of solubilized and soluble material derived from all constituents of the tumour cell. They can be effective in demonstrating cutaneous delayed hypersensitivity and in inducing lymphocyte blastogenesis [11, 18, 19] and have been used in demonstrating LMI in patients with lung cancer [6], breast cancer [20] and malignant melanoma [21]. In this study we were unable to detect activity of one 3MKCl colorectal cancer extract, but with the other two extracts prepared in a similar manner, tumour specific patterns of reactivity could be found. The reasons for these differences are unclear.

The reasons why 3MKCl extracts vary in their capacity to induce LMI are unclear, as are the reasons why slowing of migration in response to one colorectal extract appeared as inhibition compared with an unaltered control, whilst with the second extract control responses were somewhat greater than in the absence of extracts, but this variation has been observed previously [7, 22]. This variable property of the extracts, combined with the general lack of discrimination between the findings in test and control groups prevent the use of the test as a diagnostic procedure. It is also clear, from our results and those of others, that the 3MKCl extraction confers no gain in reliability or discrimination compared with simple aqueous extracts, unless a panel of such extracts is used in the manner described by Zoller *et al.* [8, 9].

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White Cell Stimulation Responses to 3MKC1 Extracts of Allogeneic Tumours in Lung and Colorectal Cancer*

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Abstract—*We have studied the stimulation responses of white cells from 71 pre-operative patients with primary lung or colorectal cancer and from 34 non-cancerous control patients. The cells were incubated with allogeneic 3MKC1 extracts of lung and colorectal cancers which we had previously shown to induce tumour-specific responses in the LMI Assay and were cultured with PHA.*

The stimulation observed in response to tumour extracts was infrequent and was not tumour specific; thus, 7 of 71 patients tested with homologous extracts showed a significant cellular response, compared with 4 of 53 patients tested with heterologous extracts. When cells from non-cancer control patients of similar age were tested with these extracts 6 of 51 responses showed significant stimulation.

We also sought to determine if pre-incubation with levamisole could enhance the frequency or specificity of cellular responses, but found that levamisole, in a concentration which significantly enhanced cancer patients' cellular responses to PHA, did not augment responses to allogeneic tumour extracts. Possible reasons why allogeneic extracts are ineffective in the stimulation assay are discussed.

INTRODUCTION

INHIBITION of leucocyte adherence to glass, of lymphocyte cytotoxicity for neoplastic cells and of leucocyte migration in the presence of neoplastic cell extracts has been generally noted in assays of cell mediated immune reactivity [1–5]. In addition, stimulatory responses have been observed in lymphocytes obtained from colorectal or lung cancer patients when the cells have been cultured with tumour cells [6] or when cultured with 3MKC1 extracts of tumours [6–8], and with crude tumour homogenates [9].

Most of such investigations have concentrated upon the responses to extracts prepared from the patient's own tumour [6, 7, 9–11] and this limitation prevents any assessment of the value of such assays as diagnostic procedures. We have therefore compared the responses of white cells from cancer patients and non-cancer controls to 3MKC1 extracts prepared from pooled allogeneic tumour tissue, using lung and colorectal cancer extracts which had both been shown to elicit tumour-specific patterns of reactivity in the leucocyte migration inhibition assay [12].

As levamisole, when administered *in vivo* or applied *in vitro* has been shown to enhance the cellular responses ot immunodepressed patients or those with malignant disease [13–15], we also sought to determine if levamisole pre-treatment would augment any responses detected.

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MATERIALS AND METHODS

White cell preparation

Twenty to twenty five millilitres of venous blood were mixed with 500 i.u. preservative-free heparin (Weddel Pharmaceuticals) in sterile glass universal bottles. Lymphocytes were obtained by centrifugation of blood samples on a gradient if Ficoll (Pharmacia, Uppsala, Sweden) and Triosil (Nyeggard & Co., Oslo, Norway) essentially by the method of Böyum [16]. Blood was diluted 1:1 with phosphate-buffered saline (PBS) pH 7.3 and 12 ml aliquots were layered onto 8 ml of an

aqueous solution of Ficoll (6.35%) and Triosil (13.4%) in 25 ml Universal bottles. The gradients were centrifuged at $500 \times g$ for 15 min, during which the granulocytes and erythrocytes sedimented through the Ficoll/Triosil phase. Mononuclear cells formed a layer between the diluted plasma and the Ficoll/Triosil phase and were collected by aspiration. The cells were then washed thrice in Eagles' Minimum Essential Medium (MEM) and then finally resuspended in Eagles' MEM supplemented with 10% heat inactivated foetal calf serum, benzyl penicillin (200 i.u./ml) and streptomycin (100 μg/ml) (Eagles 10% FCS) at a final cell density of 1.0×10^6 cells/ml or 2.5×10^6 cells/ml., according to the culture technique used. Cell viability was determined by Trypan blue dye exclusion and was always greater than 99%.

Tumour extracts

Two tumour extracts were used, a 3MKCl extract of primary lung cancer and a 3MKCl extract of colorectal cancer. The preparation and *in vitro* reactivities of each are described in the previous paper [12], the colorectal cancer extract being extract III.

Patients

White cell responses were studied in 36 hospital in-patients with lung cancer, in 35 patients with colorectal cancer, in 27 patients with non-malignant medical conditions and in 7 healthy volunteers. All the cancer patients were tested before surgery, and, in all patients, histological confirmation of the tumours was obtained. The lung and colorectal cancer patients were each of similar age (mean 64 yr, range 54–71; mean 64 yr, range 49–75 respectively) to the hospital controls (mean 62 yr, range 47–74) who had a variety of non-malignant conditions, mainly strokes, myocardial infarctions and chronic bronchitis. The healthy volunteers were members of the hospital and laboratory staff (mean age 27 yr, range 21–32).

Lymphocyte culture

(a) *Glass test tube method.* This method was based on that of Ling [17] for the mitogen stimulation of lymphoid cells. One millilitre aliquots of leucocyte suspension (1×10^6 cells/ml in Eagles 10% FCS) were added to sterile glass (Pyrex) 7.5 × 77.5 mm test tubes (Jobling glass) and tumour extracts were then added where appropriate. More Eagles—10% FCS was added to each tube to bring the final culture volume to 1.2 ml. Tubes containing medium alone and cell suspension without extracts were set up as controls. A range of concentrations of tumour extract of 1–1000 μg/ml was used. All tests were carried out in triplicate; it was usually possible to test four or five combinations of extract type and concentration. The tubes were capped with sterile, loose-fitting aluminium lids, shaken gently to mix the contents and incubated in a humidified 5% CO_2 in air atmosphere at 37°C for 4 days. Thirty microlitres of I^{125} iodoxuridine (I^{125} UdR, specific activity 50 μCi/ml) were then added to each tube. After a further 16 hr culture the tubes were centrifuged at $600 \times g$ for 15 min, the supernatant discarded and the cell pellet resuspended in 0.85% saline. The washing procedure was repeated and then the tubes with their cell pellets were counted for 5 min in a gamma counter.

(b) *Microplate method.* Microplate cell cultures were set up in which the responses of lymphoid cells to tumour extracts alone were compared with responses to PHA alone. These responses were also compared with the same responses observed when lymphoid cells had been preincubated with levamisole in phosphate buffered saline (PBS) or with PBS alone.

The method of lymphocyte culture in microplates was based on that of Dean *et al.* [18]. Aliquots of 30 or 60 μl of the tumour extracts were firstly added to replicate wells in Falcon II microplates (Sterilin), leaving empty the peripheral wells; the volumes were then increased to 100 μl by the addition of Eagles 10% FCS and then 200 μl of leucocyte suspension were added to each well. A range of concentrations of tumour extracts of 25–500 μg/ml was used. In experiments which included levamisole pre-treatment, washed lymphocytes were resuspended in 10 ml of Eagles' MEM which was then divided into two aliquots. To one of these were added 100 μl of sterile levamisole solution (10 mg/ml in PBS) to give a final concentration of 200 μg/ml, a concentration which we had found to enhance the reactivity of cancer patients' lymphocytes to PHA. To the other aliquot were added 100 μl of PBS alone. Both aliquots were incubated side by side for 1 hr in a humidified 5% CO_2 in air atmosphere at 37°C. Both aliquots were then centrifuged at $200 \times g$ for 5 min, the supernatants discarded and the cell pellets each resuspended in Eagles 10% FCS at a cell density of 2.0×10^6 cells/ml.

The cultures of cells which had been preincubated with levamisole or PBS included

replicate wells containing PHA (Wellcome Reagent grade) at a final dilution within the well of 1/100 of the reconstituted solution. The lymphocyte culture control wells contained lymphocyte suspensions with added Eagles 10% FCS instead of PHA or tumour extracts and the medium control wells contained Eagles 10% FCS alone. The empty peripheral wells were filled with medium to avoid any "edge effect" [19] and the microplates were then incubated in a humidified 5% CO_2 in air atmosphere at 37°C. After 4 days incubation 10 μl I^{125} UdR (Specific activity 50 μCi/ml) were added to each well. Sixteen hours later 230 μl of the supernatants were removed by aspiration using a needle mounted through a plastic block so as to limit the depth of the needle tip. The cells were washed twice in PBS and then resuspended in 200 μl PBS. One hundred microlitres of this suspension were then harvested onto numberd filter discs (Whatman 3M grade; 1.9 mm dia). The discs were washed twice in 10% trichloracetic acid, rinsed in methanol and then in diethyl ether and allowed to dry. The discs were each counted for 5 min in a gamma counter.

Calculations

The counts obtained from each disc or test tube were converted to counts per minute (CPM) and stimulation indices were calculated using the formula:

Stimulation Index (SI)

$$= \frac{\text{CPM cells} + \text{extract (or PHA)} - \text{CPM medium alone}}{\text{CPM cells alone} - \text{CPM medium alone}}$$

Lymphocyte stimulation by tumour extracts was regarded as significant within groups when the paired counts of stimulated and control cultures differed significantly when tested by Students *t*-test ($P<0.05$). Individual differences were arbitrarily judged as significant when the stimulation index was greater than 2·0, this criterion being the same as that adopted by others [9, 11, 20].

The effects of levamisole on responses were assessed by comparing the increments or decrements produced by levamisole over PBS pre-treatment only. The effects of levamisole were analyzed statistically using the Wilcoxon matched pair signed ranks test, calculating all probability values with two tails.

RESULTS

Figures 1 and 2 show the individual results from the test tube assay of stimulation responses to colorectal and lung tumour

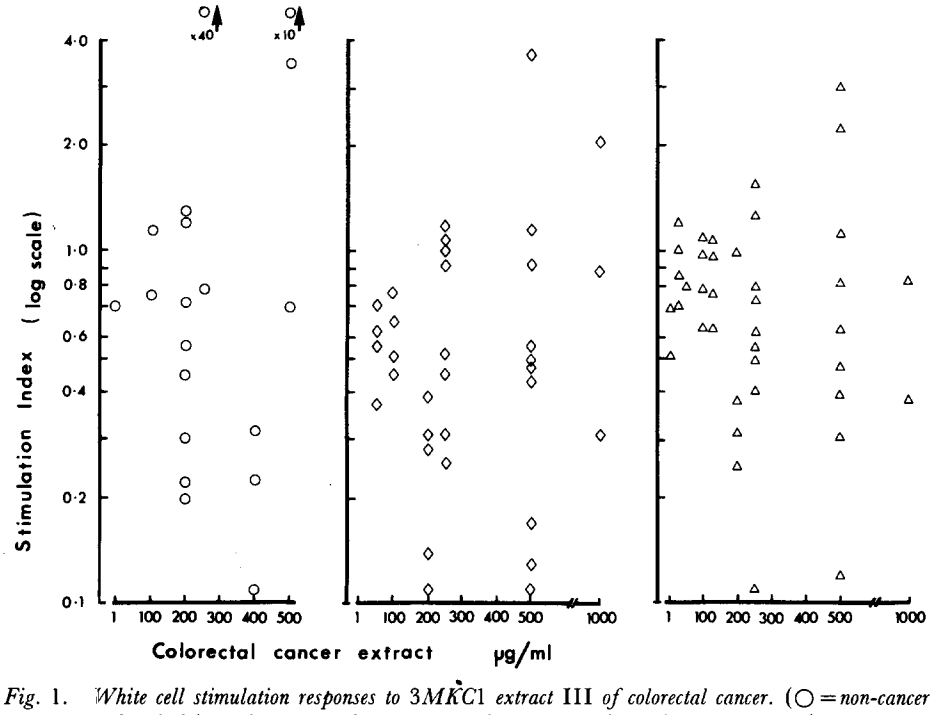

Fig. 1. *White cell stimulation responses to 3MKC1 extract* III *of colorectal cancer.* (○ = *non-cancer hospital in-patients;* ◇ = *lung cancer patients;* △ = *colorectal cancer patients.*)

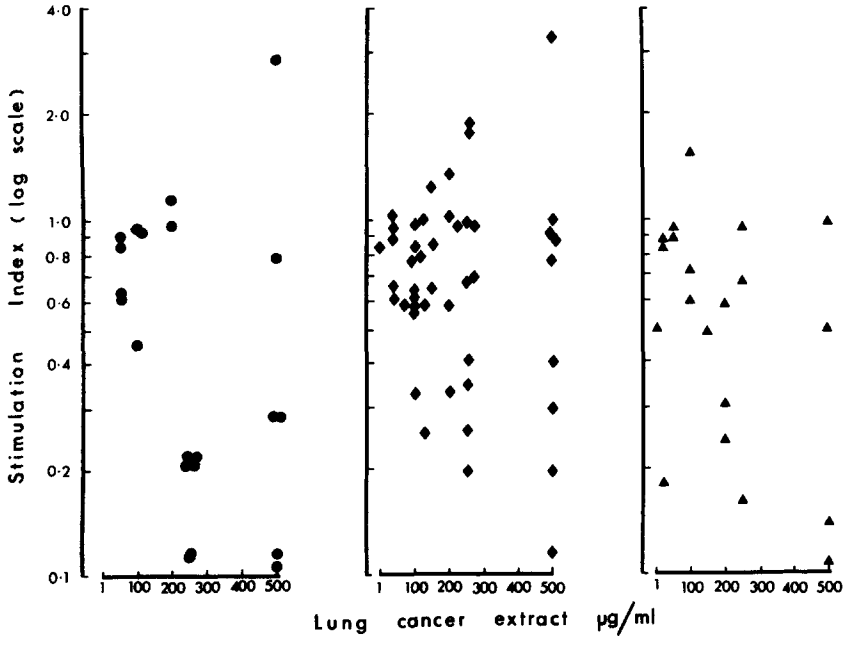

Fig. 2. White cell stimulation responses to 3MKC1 *extract of lung cancer.* (● = *non-cancer hospital in-patients;* ◆ = *lung cancer patients;* ▲ = *colorectal cancer patients.*)

Table 1. *Stimulation responses to* 3MKC1 *tumour extracts*

	No. of patients' cells stimulated (SI ≧ 2.0) / No. tested					
Culture method	Test tube		Microplate		Total	
Tumour extract	Colon	Lung	Colon	Lung	Colon	Lung
Colorectal cancer patients	2/20	0/7	2/15	1/10	4/35	1/17
Lung cancer patients	1/20	1/21	2/16	2/15	3/36	3/36
Hospital control patients	2/10	1/6	1/17	2/17	3/27	3/24

extracts in colorectal cancer and lung cancer patients and in control individuals using tumour extract concentrations of 1–1000 µg/ml. There were no significant differences in the mean values obtained with cancer patients' cells in response to homologous or heterologous tumour extracts or between the responses obtained when the cells were those of cancer bearing or non-cancerous individuals. The pattern and magnitude of the stimulation results obtained from the microplate cultures were very similar to those already illustrated.

Significant stimulation responses in individuals (defined as an index greater than 2·0) were observed on 17 occasions (Table 1) over the whole range of extract concentrations used; they occurred rather more frequently when using the microplate culture method. It is clear, however, that there was no consistent pattern in these unusual responses; thus though 7 were obtained in 71 patients in response to homologous tumour extracts, 4 were observed in 46 patients in response to heterologous extracts and 6 in 51 comparisons in hospital controls.

Effect of levamisole

Figure 3 shows the effects of levamisole pre-treatment on responses to PHA.

When lymphoid cells were incubated with levamisole prior to incubation with PHA, augmented responses to the PHA were noted in 12 of the 14 cancer patients (6 lung cancer and 6 colon cancer) studied with diminished responses in 2, as compared with enhanced responses in 8 control patients and diminished responses in 6. The difference in the cancer patients' results was statistically

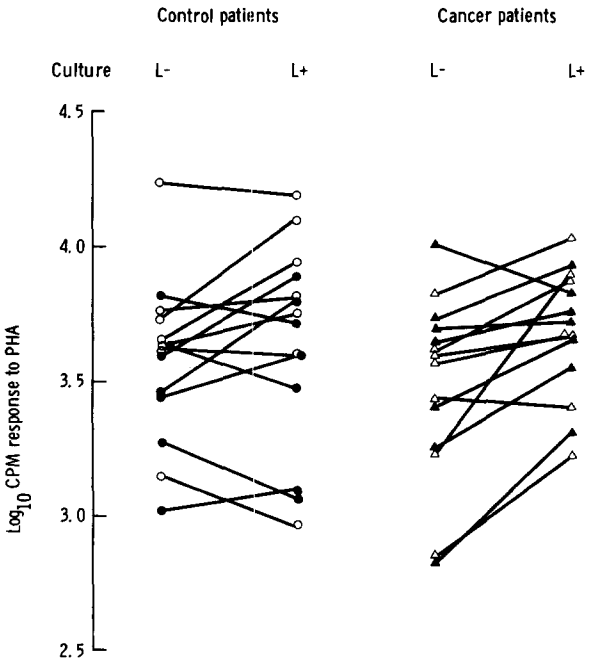

Fig. 3. The effect of pre-incubation with levamisole on stimulation responses to PHA.
(○ = healthy volunteers; ● = non-cancer hospital in-patients; △ = lung cancer patients; ▲ = colorectal cancer patients).
L^- = cultures of cells pre-incubated with PBS alone.
L^+ = cultures of cells pre-incubated with levamisole in PBS.

significant ($P<0.1$, Wilcoxon test), but not in the controls ($P>0.4$). However, when the same concentration of levamisole was used to try to augment the responses of cancer patients' cells to tumour extracts, no consistent effects were demonstrable, responses being enhanced in seven patients and diminished in seven. Table 2 summarizes these findings and shows that mean changes were virtually equivalent in the test and control groups.

DISCUSSION

We have found that when peripheral blood mononuclear cells from cancer patients and age matched non-cancer controls were incubated with allogeneic 3MKC1 extracts, stimulation responses were infrequent and did not suggest any tumour specific pattern of reactivity. These disappointing results could theoretically be due to poor culture methods or to the use of inactive tumour extracts. Our microplate culture methods were based on those of Dean *et al.* [18] for the stimulation of white cells by tumour extracts and we have used them to detect tumour specific responses in an animal model with 3MKC1 extracts of syngeneic antigenic tumours [21]. The test tube method [7] has been used in our laboratories for demonstrating responses in animal tumours [22] and is similar to those widely reported in human studies [6, 7, 20, 23].

Furthermore, we have already shown in our companion paper that the identical extracts could be used to detect specific reactivity in the LMI test [12]. Our methods could therefore have reasonably been expected to succeed if stimulation could be induced by allogeneic tumour extracts.

A third possibility is that lymphoid cells of cancer patients do not respond because of a generalized depression of reactivity as evidenced by depression of response to PHA. We think this explanation unlikely, because responses in the LMI test can be induced, but when levamisole was added to our culture media in concentrations sufficient to correct reduced responsiveness to PHA

Table 2. The effects of levamisole on leucocyte stimulation responses to tumour extracts

Stimulation responses (S.E. ± 1 S.E.M.) of patients' cells				
	Colorectal cancer extract		Lung cancer extract	
Culture	L^-†	L^+*	L^-	L^+
Colon cancer patients (7)	1.41 ± 0.19	1.13 ± 0.12	1.48 ± 0.02	1.08 ± 0.22
Lung cancer patients (7)	1.07 ± 0.32	0.95 ± 0.16	1.38 ± 0.54	1.30 ± 0.33
Hospital control patients (7)	0.85 ± 0.09	1.14 ± 0.21	0.97 ± 0.12	1.13 ± 0.18
Health volunteer (7)	0.84 ± 0.04	1.03 ± 0.08	0.87 ± 0.08	0.88 ± 0.10

$P>0.1$ in each case.
*L^+ = Cells pre-incubated with levamisole in PBS.
†L^- = Cells pre-incubated with PBS only.

there was no improvement in responses to tumour extracts. We were unable to explore the possible virtues of other concentrations of levamisole, because supplies of the active 3MKCl tumour extract were exhausted, but the absence of any possible useful trends in the levamisole responses must make a positive result at another concentration very unlikely.

Dean *et al.* [18, 24], investigating breast cancer patients, have reported similar findings to our own, namely that allogeneic 3MKCl tumour extracts which could elicit tumour specific responses in the LMI assay would only induce frequent and non-specific responses in the lymphocyte stimulation assay. These authors in discussing their results felt that histocompatibility antigens present within the extracts were the cause of the different patterns of response [24].

We have no direct evidence to bear on this conclusion, but it may be signifiacant that lymphocyte blastogenic responses to tumour cells or extracts usually require 5 or more days' culture [6, 7, 18, 20, 24], and it is this time course which is used in assays measuring the stimulation responses of unsensitized lymphocytes to histocompatibility antigens [25]. Furthermore, since it has been shown that HL-A antigens may be extracted by 3MKCl solubilisation [26], it would seem inevitable that such antigens will be present in tumour extracts and thus may mask the more subtle reactions to tumour-associated antigens.

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Evaluation of Liver and Bone Scanning in Patients with Early Breast Cancer, Based on Results Obtained from More Advanced Cancer Patients

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Abstract—*A prospective study on both bone and liver scanning was undertaken in patients with Stage* I, II *and* III *breast cancer immediately following the operation. In both series of scans the diagnostic values were evaluated in patients with more advanced breast cancer. No cases of occult metastases of the liver were identified, and* 2.3% *of* 129 *patients showed equivocal scannings with Stages* I, II *and* III *breast cancers and there were no patients with postoperative liver metastases. Ten of* 114 (8.8%) *patients with breast cancer at Stages* I, II *and* III *showed positive bone scans. However,* 6 *of them were excluded because of degenerative diseases or influences of operation, noted as the cause of a positive scan. Therefore,* 4 *of* 114 (3.5%) *patients showed abnormal positive scans which were otherwise unaccounted for. Out of these patients, only* 1 *had developed bone metastases. It may be that the bone and liver scans are restricted to patients who have a high risk of recurrence at the time of the operation, and that they could be used for the detection of early metastases during the follow-up study.*

INTRODUCTION

IT HAS been a general agreement that some of the "early breast cancers" have already widely disseminated metastases at the time of the first diagnosis. Thus, making clinical staging procedures without considering the occult metastase may be unsatisfactory [1, 2]. Furthermore, it has been interesting to see whether the scanning with technecium labelled compounds could detect clinically or roentgenographically occult metastases at the time of operation. In relation to this, there is a problem of the selection of adequate adjuvant treatments for the breast cancer. There has been much controversy concerning the evaluation of the bone scans on the above mentioned problems in patients with early breast cancer [1–11]. There were a few reports on the value of the liver scanning in the early breast cancer patients. It is more helpful to detect a liver metastasis in its early stage, because the response to treatments has been particularly poor in the later stages [12].

In the present study, both liver and bone scans were performed on patients with breast cancer at Stages I, II and III, they were evaluated for their usefullness as screening procedures to locate occult metastases. Both scans which were taken in patients with more advanced breast cancer were then compared for evaluation of the diagnostic accuracy of the scans.

MATERIALS AND METHODS

Previously untreated patients with breast cancer between September 1972 and June 1977 at the National Kyushu Cancer Center Hospital were examined and "staged" according to the UICC, TNM (1973–1977) Stage Classification. Namely, the patients were divided into three stages as follows:
Stage I

($T_{1a}N_0M_0$, $T_{1a}N_{1a}M_0$, $T_{1b}N_0M_0$, $T_{1b}N_{1a}M_0$);

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Stage II

$$(T_0N_{1b}M_0,\ T_{1a}N_{1b}M_0,\ T_{1b}N_{1b}M_0,\ T_{2a}N_0M_0,\ T_{2a}N_{1a}M_0,\ T_{2b}N_0M_0,\ T_{2b}N_{1a}M_0,\ T_{2a}N_{1b}M_0,\ T_{2b}N_{1b}M_0)$$

or Stage III (any T_3 with any N, any T_4 with any N; any T with N_2, any T with N_3, with M_0). Bone roentgenograms were routinely obtained in the regions of the skull, the cervical, thoracic and lumbar spines, the pelvis including the upper two thirds of the femurs, the ribs and the sternum. When an equivocal bone metastasis was noted on the roentgenograms or scans, the tomographies of the regions were added.

Liver scanning was performed in 129 patients with breast cancer at Stages I, II and III and in 126 patients with metastatic or advanced (Stage IV) breast cancer. Of these advanced cancer patients, 84 were laparotomized for the major endocrine ablation therapy such as adrenalectomy and were examined macroscopically relative to the presence of liver metastases.

Bone scans were obtained in 114 patients with Stages I, II and III cancer and in 110 patients with advanced or metastatic breast cancer. The scans were performed 2–4 weeks after radical operation. In more advanced cases, the scans were obtained before the beginning of the treatment. All the patients were followed up on and recurrence of malignancy was reviewed.

Liver scans were initiated 15 min after the injection of 1.5–2.0 mCi of ^{99m}technetium-phytate or -sulfur colloid, using a Toshiba GCA-II gammacamera. Anterior, posterior and right lateral views were obtained for each patient.

Bone scans were obtained by using ^{99m}technetium polyphosphonate (pyrophosphonate, diphosphonate, or methylene diphosphonate) with the varied dose between 10 and 15 mCi. Bone scans were performed 4 hr after injection of the tracer, with a Shimazu SCC-15W scintiscanner or a Toshiba GCA-II gammacamera.

RESULTS

Positive liver scans were obtained in 6 out of 84 patients with advanced (M_1) or metastatic breast cancer on whom laparotomies were performed as part of major endocrine ablation therapy, as shown in Table 1. One positive and 10 eqivocal scans were noted in patients in whom no liver metastases were shown macroscopically upon laparotomy. Positive scans were obtained in 35.7% (5 of 14) in the patients who showed liver metastases on laparotomy and there were 14.3% which had equivocal readings. When the size of a liver metastasis was less than 3 cm in diameter, the liver scans showed positive readings in one of 8 cases or 12.5%. And there was the same incidence of equivocal scans. In cases of liver metastasis over 3 cm in diameter measured on laparotomy, positive scans were noted in 4 of 6 cases (66.7%) and there were 16.7% equivocal scans.

There were no positive liver scans with 3 (2.3%) equivocal findings in 129 patients with breast cancer at Stages I, II and III (Table 2). No liver metastasis has developed during the postoperative follow up periods of 2–36 months (with an average of 17 months) in this group of patients.

The results of bone scans in patients with advanced or metastatic breast cancer

Table 1. Correlation between results of liver scanning and findings of liver metastasis noted on laparotomy in patients with advanced or metastatic breast cancer

Liver metastasis on laparotomy*	No. of patients	Liver scan Positive (%)	Liver scan Equivocal (%)
Negative	70	1 (1.4%)	10 (14.3%)
Positive	14	5 (35.7%)	2 (14.3%)
less than 3 cm in diameter	8	1 (12.5%)	1 (12.5%)
more than 3 cm in diameter	6	4 (66.7%)	1 (16.7%)
Total	84	6 (7.1%)	12 (14.3%)

*Liver metastases were checked macroscopically on laparotomy, most of which were confirmed histologically.

Table 2. Results of liver scanning in patients with primary breast cancer (Stages I, II and III)

Stage	No. of patients	Liver scan Positive (%)	Liver scan Equivocal (%)
I	14	0	0
II	68	0	2 (2.9%)
III	47	0	1 (2.1%)
Total	129	0	3 (2.3%)

Table 2a. *Results of liver scanning in patients with primary breast cancer* (*Stages* I, II *and* III)

N classification	T classification T1a	T1b	T2a	T2b	T3a	T3b	T4a	T4b	T4c	Total
N 0	0/8 (0/8)	0/1 (0/1)	0/25 (1/25)	0/1 (0/1)	0/5 (0/5)	— —	— —	0/1 (0/1)	0/1 (0/1)	0/42 (1/42)
N 1a	0/5 (0/5)	— —	0/18 (1/18)	— —	0/2 (0/2)	0/1 (0/1)	— —	0/4 (0/4)	— —	0/30 (1/30)
N 1b	0/4 (0/4)	— —	0/20 (0/20)	— —	0/7 (0/7)	— —	0/1 (0/1)	0/3 (0/3)	— —	0/35 (0/35)
N 2	— —	— —	0/8 (0/8)	— —	— —	0/1 (0/1)	— —	0/8 (0/8)	0/2 (0/2)	0/19 (0/19)
N 3	0/1 (1/1)	— —	— —	— —	— —	— —	— —	0/2 (0/2)	— —	0/3 (1/3)
Total	0/18 (0/18)	0/1 (0/1)	0/71 (2/71)	0/1 (0/1)	0/14 (0/14)	0/2 (0/2)	0/1 (0/1)	0/18 (0/18)	0/3 (0/3)	0/129 (3/129)

Numbers in parenthese show the equivocal readings.

Table 3. *Correlation between results of bone scanning and roentgenograms in patients with advanced or metastatic breast cancer*

Bone roentgenogram	No. of patients	Bone scan Positive (%)	Equivocal (%)	Negative (%)
Positive	75	62 (82.7%)	7 (9.3%)	6 (8.0%)
Negative	35	11 (31.4%)	5 (14.3%)	19 (54.3%)
Total	110	73 (66.4%)	12 (10.9%)	25 (22.7%)

were compared with those of bone roentgenograms (Table 3). Out of 110 patients with advanced cancer, 75 patients had distinct bone metastases revealed on roentogenograms. In these patients, 62 or 82.7%, had positive bone scans, 7 or 9.3% had equivocal scans and 6 or 8.0% of the patients had negative scans. Therefore, when the equivocal scans were included in the negative group, the true positive rate of bone scans were shown to be 82.7%, with 17.3% of false negative rate on the basis of bone roentgenograms. On the contrary, in 35 patients without bone metastases on roentgenograms, bone scans showed 11 (31.4%) positive, 5 (14.3%) had equivocal findings with 19 (54.3%) having negative scans, thus showing 31.4% false positive rate and 69.6% of true negative rate. Thus in the diagnosis of bone metastases by bone scans as compared with roentgenography, there were less false negative cases and higher incidence of false positive cases.

In this study on the bone scans in patients with early breast cancer, we firstly checked the abnormal accumulation of isotope on the scans without knowing the results of bone survey in the afore-mentioned regions. These tentative results were shown in Table 4.

Table 4. *Results of bone scanning in patients with breast cancer at Stages* I, II *and* III

Stage	No. of patients	Bone scan Positive (%)	Equivocal (%)
I	14	1 (7.1%)	2 (14.3%)
II	66	7 (10.6%)	8 (12.1%)
III	34	2 (5.9%)	6 (17.6%)
Total	114	10 (8.8%)	16 (14.0%)

Positive bone scans were obtained in 8.8% or 10 of 114 patients with breast cancer at Stages I, II and III. Fourteen per cent or 16 patients showed equivocal scans. The positive rates in the groups of Stages I, II and III were more or less similar.

Table 4a. *Results of bone scanning in patients with breast cancer at Stages* I, II *and* III

N classification	T classification T1a	T1b	T2a	T2b	T3a	T3b	T4a	T4b	T4c	Total
N 0	0/8 (1/8)	0/1 (0/1)	2/22 (3/22)	0/1 (0/1)	0/6 (3/6)	— —	— —	0/1 (0/1)	0/1 (0/1)	2/40 (7/40)
N 1a	1/5 (2/5)	— —	2/17 (1/17)	— —	0/2 (0/2)	0/1 (1/1)	— —	0/2 (1/2)	— —	3/27 (5/27)
N 1b	0/5 (1/5)	— —	2/21 (2/21)	— —	2/6 (0/6)	— —	0/1 (0/1)	0/1 (0/1)	— —	4/34 (3/34)
N 2	— —	— —	1/5 (1/5)	— —	— —	0/1 (0/1)	— —	0/5 (0/5)	0/1 (0/1)	1/12 (1/12)
N 3	— —	— —	— —	— —	— —	— —	— —	0/1 (0/1)	— —	0/1 (0/1)
Total	1/18 (4/18)	0/1 (0/1)	7/65 (7/65)	0/1 (0/1)	2/14 (3/14)	0/2 (1/2)	0/1 (0/1)	0/10 (1/10)	0/2 (0/2)	10/114 (16/114)

Numbers in parentheses show the equivocal readings.

The recurrence of malignancy was investigated in 114 patients with primary breast cancer according to the results of the bone scans occurring during periods lasting from 2 to 52 months (with an average of 13 months) after the radical operation (Table 5). In 10 patients with positive bone scans there was one patient with bone metastases. Two of 16 patients with equivocal scans had shown the recurrence; however, the sites were not osseous. Recurrences not related to the bone had occurred in 4 of 88 patients with negative scans during the post-operative course. This may suggest that there are some false positive cases in the bone scans in early breast cancer patients, although some small parts of the positive scans indicate a real bone metastasis of breast cancer. This may partly come from the unspecificity in the accumulation of the radioisotope on the sites of cancerous metastases. We observed, among 15 cases with benign diseases investigated in this hospital by bone scanning, that 4 cases or 26.7% were positive and 5 or 33.3% were equivocal.

Therefore, some factors that may influence the positive nature of the scans were studied. The distribution of the positive accumulation of the tracer isotopes including the equivocal one was compared in the following three groups: 1. patients with Stages I, II and III breast cancer (114 cases); 2. patients with recurrent or advanced breast cancer whose bone metastases could not be confirmed roentgenographically (35 cases); 3. patients with distinct bone metastases (75 cases) (Table 6). Higher incidences of abnormal isotope accumulation in the early breast cancer group or in the advanced cancer group not related to bone metastases were concentrated in the lumbar spines and pelvis, and to the operation sites such as the sternum, ribs and clavicle. Equivocal scans

Table 5. *Recurrence of breast cancer in patientst at Stages* I, II *and* III *according to the results of the bone scans*

Bone scans	No. of cases	Mean follow up period (months)	Range (months)	Metastasis to bone	Metastasis other than bone
Positive	10	10.3	3–16	1	0
Equivocal	16	14.0	4–52	0	2
Negative	88	13.5	2–38	0	4
Total	114	13.2	2–52	1	6

Table 6. *Distribution of sites of positive and equivocal scans in* 3 *groups of patients*

Site of positive or equivocal scans	I Patients with breast cancer at Stages I, II and III	II Recurrent patients without bone metastases	III Patients with bone metastases
No. of cases	10 (16)	11 (5)	62 (7)
No. of sites	12 (18)	35 (12)	165 (18)
skull	1 (3)	3 (4)	18 (5)
sternum	2 (1)	10 (0)	18 (0)
ribs, clavicle	2 (4)	6 (2)	20 (5)
scapula	0 (1)	1 (0)	3 (0)
cervical spines	1 (0)	1 (0)	7 (1)
thoracal spines	1 (0)	2 (0)	24 (2)
lumbar spines	3 (5)	6 (3)	33 (4)
pelvis	2 (3)	4 (2)	27 (0)
extremities	0 (1)	2 (0)	15 (1)

Numbers in parentheses show the number of patients with equivocal scans.

were dominant also in these regions and including the skull. This suggests that false positive bone scans may have occurred in these regions.

We tried to find whether benign diseases or operable procedures had an effect on the positive nature of the scan in these regions studied. As shown in Table 7, the presence of osteoporosis and/or spondylosis deformans was examined on the lumbar spines with two direction roentgenograms and it was compared with the results of the bone scans in the examined region. When the roentgenograms exhibited positive signs of osteoporosis and/or spondylosis, we took positive bone scans in 3 out of 53 cases or 5.7%, with the same percentage of equivocal scans. On the contrary, in the negative signs of these benign changes, only equivocal scans were noted in 2 of 61 (3.3%). This difference indicates that some, if not all, of these benign diseases had an influence on the positive nature of the bone scans in the lumbar spines. If these positive scans probably due to the benign diseases, were excluded from the total positive number of cases, the positive scans would be reduced to 6.0% (7 of 114) with 11% (13 of 114) equivocal scans in the breast cancer patients.

The Halsted radical mastectomy and an extended operation which included parasternal lymph nodes dissection following the resection of the cartilages of 2nd, 3rd and 4th ribs were performed as a standard method for the Stages I, II and III breast cancer in our hospital. The relationship between the operation method (resection of cartilages) and the bone scans were studied, comparing the two methods (Table 8). Three (4.1%) positive and 5 (6.8%) equivocal scans were shown in 73 patients subjected to the extended radical mastectomy. Radical mastectomy alone did not influence the positive nature of the scans. If these positive scans, probably due to the resection of ribs were excluded, positive scans would be reduced to 3.5% (4 of 114) with 8.8% (10 of 114) of equivocal scans (Table 9).

This incidence may be maximal, because some cases in the positive scans may be excluded from the true positive due to some unknown benign changes. Only 1 patient had developed bone metastases postoperatively in this group, and the remaining 3 patients with positive scans had not yet

Table 7. *Relation of the presence of osteoporosis and/or spondylosis deformans on the lumbar spines to the results of bone scanning*

Osteoporosis and/or spondylosis deformans	No. of patients	Bone scan Positive (%)	Bone scan Equivocal (%)
Positive	53	3 (5.7%)	3 (5.7%)
Negative	61	0	2 (3.3%)
Total	114	3 (2.6%)	5 (4.4%)

Table 8. *Relation of the operation methods of breast cancer to results of bone scans in the parasternal region*

Operation method	No. of patients	Bone scan Positive (%)	Bone scan Equivocal (%)
Extended radical mastectomy (parasternal disection)	73	3 (4.1%)	5 (6.8%)
Radical mastectomy	41	0 (0%)	0 (0%)

Table 9. *Corrected results of bone scans in patients with breast cancer at Stages* I, II *and* III

Stage	No. of cases	Bone scans Positive (%)	Equivocal (%)
I	14	0	1 (7.1%)
II	66	2 (3.0%)*	3 (4.5%)
III	34	2 (5.9%)	6 (17.6%)
Total	114	4 (3.5%)	10 (8.8%)

The benign diseases were excluded.
*One of the patients developed bone metastases.

breast cancer using ^{198}Au colloid, 68.2% (15/22) true positive rate and 2.2% (1/46) false positive rate were obtained [13]. However, it has been recognized that liver scanning is more sensitive in demonstrating the abnormality than combined liver function tests and a clinical estimation of hepatomegaly [12, 14].

No occult metastases of the liver were identified with 2.3% of the equivocal readings in 129 patients in whom liver scanning was performed at the time of operation (Table 2). As there were no patients with

Table 9a. *Corrected results of bone scanning in patients with breast cancer at Stages* I, II *and* III

N classification	T classification T1a	T1b	T2a	T2b	T3a	T3b	T4a	T4b	T4c	Total
N 0	0/8 (0/8)	0/1 (0/1)	0/22 (0/22)	0/1 (0/1)	0/6 (3/6)	— —	— —	0/1 (0/1)	0/1 (0/1)	0/40 (3/40)
N 1a	0/5 (1/5)	— —	0/17 (1/17)	— —	0/2 (0/2)	0/1 (1/1)	— —	0/2 (1/2)	— —	0/27 (4/27)
N 1b	0/5 (0/5)	— —	2/21 (2/21)	— —	2/6 (0/6)	— —	0/1 (0/1)	0/1 (0/1)	— —	4/34 (2/34)
N 2	— —	— —	0/5 (1/5)	— —	— —	0/1 (0/1)	— —	0/5 (0/5)	0/1 (0/1)	0/12 (1/12)
N 3	— —	— —	— —	— —	— —	— —	— —	0/1 (0/1)	— —	0/1 (0/1)
Total	0/18 (1/18)	0/1 (0/1)	2/65 (4/65)	0/1 (0/1)	2/14 (2/14)	0/2 (1/2)	0/1 (0/1)	0/10 (1/10)	0/2 (0/2)	4/114 (10/114)

Numbers in parentheses show the equivocal readings.

shown the signs of bone metastases clinically or roentgenographically.

DISCUSSION

The value of bone and liver scans at the time of the initial treatment was determined in patients with early breast cancer. The diagnostic value of both scans was evaluated in patients with more advanced breast cancer. Fifty per cent of the patients who showed liver metastases on laparotomy were positive on the liver scans including the equivocal ones, with a 15.7% false positive rate (Table 1). Especially in the early stages, the liver metastasis could be identified only in 12.5% of the patients. The liver scans with ^{99m}Tc-phytate of -sulfur colloid did not surpass the scans using ^{198}Au colloid in the detection of metastasis. Namely, in our investigation of liver metastases from postoperative liver metastasis in this group, the estimation of liver scans as a detector of metastasis is still uncertain. However, the investigation of liver metastasis by scans may be too expensive to be performed in all patients with early breast cancer, considering the low ability to detect an early metastasis of the liver from the breast cancer.

It has been widely accepted that the bone scanning is more sensitive than radiography for the detection of early skeletal metastases of the breast cancer [1]. In the present investigation within 110 patients with recurrent breast cancer, there were 17.3% false negative cases and 31.4% false positive cases, as compared to the roentgenograms, when the equivocal scans were considered as negative (Table 3). Bone scans, thus inherently, have a high incidence of recognized false positive abnormalities. This

relative non-specificity of the isotope accumulation is perhaps rather controversial concerning the incidence of positive scans and the predictability of the future bone metastases. Many investigators have noted a higher incidence of positive scans in about 10–40% of the patients with early breast cancer and most of them emphasized a higher incidence of development of bone metastases in the patients postoperatively [1–5]. Sklaroff and Sklaroff [6], however, showed that patients with positive scans had a slight but statistically significant increase in incidence of the metastatic disease, particularly in the bone, but that 40% of the patients with positive scans and negative roentgenograms survived 8 yr without evidence of any metastatic disease. In the present study, 10 out of 114 (8.8%) patients with Stages I, II and III breast cancer have showed positive scans (Table 4). However, 6 of these patients can be excluded, because of reasons other than metastases, such as degenerative diseases (Table 7) and the influences of operation (Table 8) [15] could be shown as the cause of the positive nature of the scan. Only 4 of 114 (3.5%) patients showed abnormal scans, otherwise unexplainable (Table 9). This percentage is equivalent to those reported recently, [7–9] and much lower than others reported elsewhere [1–5]. Among them only 1 patient had developed bone metastases in the part of the sites indicated by the scan at the time of the radical operation. Al-Domeiri and Shroff [10] and Baker *et al.* [11] showed that there were much higher percentages of the positive scans in Stage III breast cancer patients than those with Stages I and II breast cancer. Our results are not so demonstrative as theirs, but the higher frequency of equivocal scans in patients with breast cancer at Stage III may be considered as having a similar tendency (Table 9).

Our study is even now in progress and further follow up studies will be necessary to determine the value of the scans in the patients. Although there are certainly a few patients who already have metastases at the initial diagnosis of early breast cancer, it would be too expensive to examine all patients with bone scans.

It may be more likely that the bone and liver scans are restricted only to breast cancer patients with high risk of recurrence and to postoperative patients for the early detection of metastases during the follow-up study.

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Genetic Control of Endogenous Murine Mammary Tumour Viruses Reinvestigated*

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Abstract—*Milk samples of progeny mice from crosses between mouse strains which differ in the degree of release of endogenous murine mammary tumour virus (MuMTV) were tested for the presence of MuMTV antigens by means of the Sepharose bead immunofluorescence assay. The results indicated that as compared to the nonexpressor strain BALB/c, a single dominant gene controls the release of a MuMTV in the C3Hf strain. However, the low-cancer strain C57BL carries a dominant, epistatic gene which can inhibit virus release in the hybrid with C3Hf to a large extent. The GRS strain harbours a dominant gene which, in F1 hybrids of all mouse strains tested, causes the release of large quantities of a virus (MuMTV-P) which causes tumours at an early age. Other genetic factors control the release of a lesser oncogenic virus in the GRS strain. The animals tested for MuMTV antigens were subsequently forcibly bred for mammary carcinoma production. There is a good correlation between virus release at a young age and tumour development at a late age, but a substantial number of mice which were virus-negative nevertheless developed a mammary tumour.*

INTRODUCTION

GENETIC transmission of murine mammary tumour virus (MuMTV) was initially concluded from the observed genetic control of gamete-borne transmission of the virus in several mouse strains [1–5]. It was assumed that genetic information (germinal provirus, [6]) for a MuMTV would be present not only in those mouse strains which spontaneously release a gamete-borne MuMTV but also in low cancer strains. In the latter strains, expression, i.e. transcription of the germinal provirus, would be repressed. This view was substantiated by the finding that every mouse strain tested so far carried multiple copies of MuMTV homologous DNA, irrespective of virus expression and mammary carcinoma incidence [7–10], but only the high cancer strains would contain large amounts of MuMTV-RNA in various tissues [11].

From crosses between nonexpressor strains and MuMTV-releasing strains, it was deduced that single Mendelian factors control the expression of the virus [1–5, 12–15]. In the case of the peculiar mouse strain GRS, which produces tumours at a very early age under the influence of a genetically transmitted MuMTV [16], the conclusion of a single dominant gene controlling the release of the highly oncogenic virus was based mainly on the study of tumour incidences [1–5, 12, 13]. This conclusion has been seriously challenged [17–19], in particular on the basis of detection of viral antigens in the milk by means of a sensitive immunoassay.

Since the sensitive Sepharose bead immunofluorescence assay had been perfected [20, 21] and applied to the detection of oncoviral antigens [22, 23] in our laboratories, it was decided to reinvestigate the genetic control of the release of endogenous MuMTV with this method in addition to tumour detection.

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MATERIALS AND METHODS

Mice

The following mouse strains were used: 1. the high mammary cancer strains GRS and BALB/cfC3H. 2. the moderate mammary cancer strains C3Hf and BALB/c. 3. the low cancer strain C57BL.

Female mice, either inbred or hybrid, were caged at 4 months of age with a 3-month-old (C57BL × BALB/c) F1 male. Two weeks post-

partum the female was milked (0.25–0.5 ml) and the litter discarded. The defatted milk was stored at −30°C until tested in the immunoassay. The female was earmarked and then caged with two other females and a (C57BL ×BALB/c)F1 male. The cages were examined daily for litters, which were discarded. The males were replaced by a 3-month-old male every four months. The females were inspected twice a week for the presence of mammary tumours or other diseases. They were permitted to live out their life spans.

Virus

The standard strain of mammary tumour virus (MuMTV-S) [3] was isolated from BALB/cfC3H mammary tumours according to Westenbrink *et al.* [24]. This method is a modification of the technique developed by Calafat and Hageman [25]. In brief, ultrapellets of cell-free homogenates are first centrifuged on a discontinuous sucrose gradient and thereafter twice on a linear (20–50% w/w) sucrose gradient.

The endogenous, virulent MuMTV-O strain isolated from old BALB/c mice [26], had been kindly provided in 1972 by Dr. Ph. C. Hageman of the Netherlands Cancer Institute. After inoculation, a BALB/c colony which continuously transmits this virus through the milk and which develops a high incidence of mammary carcinomas at a young age was established. Virus was isolated from tumours as described above.

The so-called low oncogenic variant (MuMTV-L, [3]) was isolated from transplantable C3Hf mammary tumour lines. The virulent variant MuMTV-P was isolated from primary GRS tumours.

For bioassay, the virus was diluted in Hanks' balanced salt solution and 5 μg in 0.5 ml were then immediately injected into 4-week-old female BALB/c mice, which were then subjected to forced breeding [3].

Antiserum

A rabbit was immunized with 0.2 mg of purified MuMTV-S emulsified in complete Freund's adjuvant at multiple subcutaneous and intramuscular sites. This immunization was repeated two weeks later and the rabbit was bled two weeks thereafter. Antibodies to murine host materials were removed by immunoabsorbent column chromatography [27] using Sepharose-4B beads (Pharmacia, Uppsala, Sweden) coupled with either normal BALB/c mouse serum or normal BALB/c mammary gland extract.

In the Sepharose bead immunofluorescence assay [20–23], the antiserum was tested for reactivity with MuMTV, murine leukaemia virus, normal mouse serum, mouse mammary gland extract and ovalbumin. The antiserum proved to be satisfactorily specific.

An immunoglobulin fraction of the absorbed antiserum was prepared by DEAE-cellulose chromatography [28]. A portion of this fraction was coupled to Sepharose-4B beads according to March *et al.* [29]. Another portion of this fraction was labelled with fluorescein isothiocynate (FITC) [30].

Sepharose bead immunofluorescence assay for the detection of MuMTV antigens

The method is essentially the same as developed for C-type oncoviral antigens [22]. The Sepharose beads coupled with the antiserum to MuMTV was pipetted into wells of flat bottom microtitration plates (Cooke System, no. M220-24A, Greiner, Nürtingen, Germany). Each well contained 50 μl of a 1% (v/v) suspension.

The skimmed milk samples were diluted in such a way that they contained 0.5 mg protein per ml. The protein content was determined by the fluoram method [31]. The samples were then incubated with 0.3% Nonidet (NP40, Shell Oil Co., The Hague, The Netherlands). Two-fold dilutions of these milk samples (50 μl) in PBS were added to the wells along with beads coupled with antiserum. After 1 hr incubation, the beads were washed four times with PBS, using a homemade apparatus. They were then incubated for 1 hr with the FITC-conjugated antiserum fraction, which was diluted 50-fold in PBS with 2% hemoglobin.

Individual bead fluorescence was then measured with an inverted Diavert microscope (Leitz, Wetzlar, Germany) using a 25 × 0.50 water immersion objective. The microscope was equipped with an MPVI measuring device and a Ploemopak 2.2 incident light illuminator. The Volt meter (Hewlett Packard no. 3490, Palo Alto, California, USA), reading the photomultiplier (S20 cathode sensitivity) output was interfaced directly to a desk top programmable calculator (Hewlett Packard, no. 9821A) which immediately provided the average fluorescence and its standard error of ten beads per well.

The amount of viral antigens in milk samples was determined on the basis of a calibration curve obtained for purified MuMTV-S.

RESULTS

Mammary tumour incidences in mouse strains and their hybrids used in this investigation

For characterization with regard to mammary tumour development, relatively large numbers of animals from the various strains and their hybrids were forced bred. In addition, the efficacy of purified MuMTV preparations of different origin were compared in the susceptible BALB/c mouse strain.

It can be concluded from Table 1 that tumours appear extremely early in the GRS strain, which produces the genetically transmitted MuMTV-P. These lesions usually regress after parturition, but gradually become pregnancy-independent. Tumours appear somewhat later in the BALB/cfC3H strain, which carries exogenous MuMTV-S. These tumours sometimes show a partial regression after parturition, but during the next pregnancy they become fully autonomous.

The C3Hf mouse strain, which releases a lesser oncogenic MuMTV, shows a moderate tumour incidence (30%) at 18 months of age. Only one female of the C57BL strain, which does not seem to release a MuMTV, developed a mammary tumour. The BALB/c strain, which produces small amounts of the virulent MuMTV-O at a late age, develops a substantial number of tumours at 18 months of age like the C3Hf strain.

Infection of BALB/c mice with any of the MuMTV-strains results in a very high tumour incidence. The MuMTV-P strain isolated from GRS tumours is slightly less virulent than MuMTV-S and -O. The MuMTV-L strain isolated from C3Hf tumours is considerably less virulent, in that it induces tumours at a significantly later age. The incidence is surprisingly high, however.

All hybrids sired by a GRS male show an approximately 100% tumour incidence. The hybrid (BALB/c × GRS)F1 develops these lesions as early as does the parental GRS, while the hybrids of C3Hf or C57BL with GRS have a significantly later tumour age. In the two latter hybrids, tumours show a shorter period of pregnancy-dependency.

While the hybrid of C57BL and C3Hf has a tumour incidence comparable to that of the C3Hf strain, the combination of BALB/c and C3Hf results in a surprisingly high incidence (63%) as compared to the parental strains. The latter hybrid develops tumours at about the same age as C3Hf, whereas (C57BL × C3Hf)F1 shows tumours considerably later. The hybrid of BALB/c and the low cancer strain C57BL has a moderate tumour incidence like BALB/c, but neoplasms arise 7 months later on the average.

Immunoassay of milk samples for MuMTV antigens

When purified MuMTV preparations or tissue culture fluids were assayed with the Sepharose bead immunofluorescence assay, the lowest detectable amount of viral proteins was 0.3 ng per sample of 50 μl. Probably because of nonspecific binding of milk proteins to the beads, the sensitivity is considerably less for mouse milk: 5 ng per sample of 50 μl. The tested milk samples can be divided into three separate groups: (a) negative; (b) less than 5 μg MuMTV antigens per mg protein; (c) more than this amount. The incidences of the three groups for milk samples of the various mouse strains and their hybrids are given in Table 2.

The high cancer strains GRS and BALB/cfC3H have high levels of virus in their milk: the averages are 70 μg and 50 μg, respectively, per 1 mg milk protein. The C3Hf mouse strain produces considerably less: 10 μg on the average. The strains C57BL and BALB/c are negative for the virus.

The F1 hybrids produced by a GRS male had high levels of MuMTV proteins in their milk. Most (C57BL × C3Hf)F1 animals are MuMTV-negative, while all animals in the cross BALB/c × C3Hf are positive. They produce approximately twice as much MuMTV proteins as does C3Hf.

Except for one animal, the F1 hybrid of the "virus-free" mouse strains C57BL and BALB/c does not release MuMTV into the milk.

The results of the immunoassays of milk samples from progeny mice of the various series of crosses are presented in Table 3. The first five crosses concern the genetics of the release of MuMTV in the GRS strain and its hybrids. In all three crosses in which the C57BL strain is involved, incidences of MuMTV-positive milk samples are close to 50%. In those crosses in which the C57BL is not involved, the incidences are very much in excess of the anticipated 50%. However, when the incidences of the group with more than 5 μg are considered, they are around 50%, except for the cross ♀BALB/c × ♂(C3Hf × GRS)F1.

The next five crosses concern the genetics of release of MuMTV in the C3Hf strain and its hybrids. The incidences in the first

Table 1. Mammary tumour development in inbred mouse strains and their F1-hybrids

Mouse strain or F1 hybrid*	Number of animals	Number with mammary tumour	Percentage	Average tumour age in months ±S.D.
GRS	154	154	100	4.3±1.0
BALB/cfC3H	442	430	97	6.4±0.9
C3Hf	90	28	31	17.9±3.1
C57BL	44	1	2	21.5
BALB/c	90	27	30	18.0±3.7
BALB/c+MuMTV-S†	158	140	89	10.7±3.2
BALB/c+MuMTV-O	125	93	74	10.8±4.1
BALB/c+MuMTV-P	60	45	75	12.3±2.7
BALB/c+MuMTV-L	50	38	76	18.8±4.3
C3Hf×GRS	110	109	99	7.7±2.5
C57BL×GRS	140	137	98	6.3±2.1
BALB/c×GRS	98	98	100	4.8±1.6
C57BL×C3Hf	52	13	25	21.4±4.6
BALB/c×C3Hf	64	40	63	17.0±3.6
C57BL×BALB/c	76	23	30	24.7±3.8

*F1 hybrids were sired by the second indicated strain.
†5 μg of purified virus was injected intraperitoneally into 4-week-old mice.

Table 2. MuMTV antigens in milk samples of inbred mouse strains and their F1-hybrids

Strain or hybrid	Number of animals	Number negative	Number with less than 5 μg	Number with more than 5 μg	Total incidence MuMTV-positives (%)
GRS	21	0	1	20	100
BALB/cfC3H	17	0	0	17	100
C3Hf	14	0	2	12	100
C57BL	28	28			0
Balb/c	24	24			0
C3Hf×GRS	27	0	4	23	100
C57BL×GRS	31	0	5	26	100
BALB/c×GRS	36	0	3	33	100
C57BL×C3Hf	28	24	3	1	14
BALB/c×C3Hf	31	0	9	22	100
C57BL×BALB/c	38	37	1		3

Table 3. MuMTV antigens in milk samples of progeny of several crosses

Cross No.	Female	F1-hybrid male*	Number of animals	Number MuMTV negative	Number with <5 μg MuMTV†	Number with >5 μg MuMTV†	Incidence (%) MuMTV positives
1	C57BL	C57BL×GRS	166	82	3	81	51
2	C57BL	BALB/c×GRS	48	20	2	26	58
3	BALB/c	BALB/c×GRS	103	15	39	48	85
4	BALB/c	C57BL×GRS	135	56	18	61	59
5	BALB/c	C3Hf×GRS	192	36	8	148	81
6	BALB/c	BALB/c×C3Hf	277	156	5	116	44
7	C3Hf	BALB/c×C3Hf	104	9	6	89	91
8	C3Hf	C57BL×C3Hf	111	51	6	54	54
9	C57BL×C3Hf	C57BL×C3Hf	68	54	1	13	21
10	BALB/c	C57BL×C3Hf	84	67	2	15	20

*F1 hybrids were sired by the second indicated strain.
†Amount of MuMTV antigens per mg of protein in the milk samples.

four crosses would be according to a single gene hypothesis: the difference would be that, in the case of BALB/c versus C3Hf, a dominant C3Hf gene would control virus release, while that gene seems to be recessive in the crosses involving the C57BL strain. From the cross BALB/c × (C57BL × C3Hf)F1 with its 20% incidence it seems that at least two genes are involved.

Tests for extrachromosomal male transmission of MuMTV

In the crosses involving GRS-F1 hybrid males, the females were mated only once to these males in order to avoid extrachromosomal male transmission of MuMTV [1, 2]. The females were then mated with a BALB/c male and allowed to raise a litter. In the meantime, they were milked once for immunoassay of MuMTV antigens. They were then subjected to forced breeding. The females of the litter sired by a BALB/c male were also tested for the presence of MuMTV antigens in their milk and afterwards subjected to forced breeding.

In cross No. 1, one C57BL female was shown to be infected on the basis of immunoassay. However, she did not develop a mammary tumour, but died of a lymphoreticular neoplasm at the age of 20 months. Of her (C57BL × BALB/c)F1 offspring, all three females proved to be positive for viral antigens in the milk. Two of them produced a mammary carcinoma at around 1 yr of age (11 and 13 months, respectively).

In cross No. 3, four BALB/c females proved to be antigen positive. Only two of them developed a tumour before one year of age, while the total mammary tumour incidence in this group was 28% with an average tumour age of 15.0±1.1 months. The offspring of these 4 females also proved to be infected on the basis of immunoassays and the fact that the majority of the progeny females produced a tumour before 1 yr of age.

In crosses 4 and 5 it was also found that not every infected BALB/c female developed an early mammary tumour. In cross No. 4, one infected female died from a lymphosarcoma at 16 months. In cross No. 5, one antigen positive female developed a mammary tumour at 19 months and one accidently drowned at the age of 15 months.

In crosses 4 and 5, some litters sired by a BALB/c male were positive with regard to MuMTV antigens in their milk as well as to early tumour development, whereas their mothers proved to be negative in both tests.

In conclusion, only 1% of the C57BL females seemed to be infected by a single mating with a GRS-hybrid male, whereas approximately 10% of the BALB/c females contracted the virus from their GRS-hybrid mates.

Mammary tumour development in mice assayed for MuMTV antigens

The mammary tumour incidences in forced bred females of the various inbred mouse strains and their F1 hybrids in relation to their MuMTV status is given in Table 5.

As compared to Table 1 in which considerably larger numbers of animals were used, the incidence and average tumour ages of the samples used in Tables 5 are not substantially different from those of the corresponding groups in Table 1.

Tumour incidences are significantly higher and tumour ages substantially lower in the classes in which MuMTV antigens can be detected at first lactation. There is also a slight difference in this respect between the low-MuMTV-releasers (less than 5 μg) and the high-MuMTV-releasers. For instance,

Table 4. Tests for extrachromosomal male transmission of MuMTV in crosses involving GRS hybrid males

Cross No.	Strain female mated to GRS-F1 hybrid	Number of females	Number of MuMTV-antigen positives	Number with early tumours*	Number with MuMTV-positive litters†
1	C57BL	61	1	0	1
2	C57BL	20	0	0	0
3	BALB/c	36	4	2	4
4	BALB/c	53	4	3	6
5	BALB/c	90	7	5	8

*Mammary tumours appearing before 1 yr of age.
†Based on immunoassay and early mammary tumour development.

Table 5. Mammary tumour incidences in inbred mouse strains and their hybrids in relation to MuMTV status

Mouse strain or F1 hybrid	Number of animals	MuMTV-negative		<5 μg MuMTV per mg protein		>5 μg MuMTV per mg protein		Total	
		Tumour %	Tumour age months	Tumour %	Tumour age months	Tumour %	Tumour age months	Tumour %	Tumour age months
GRS	21			100	8	100	6	100	6
BALB/cfC3Hf	17					100	8	100	8
C3Hf	14			0		58	18	50	18
C57BL	28	0						0	
BALB/c	24	21	22					21	22
C3Hf × GRS	27			100	11	100	6	100	8
C57BL × GRS	31			100	9	100	6	100	7
BALB/c × GRS	36			100	8	100	6	100	6
C57BL × C3Hf	28	13	26	100	22	100	18	25	23
BALB/c × C3Hf	31			33	20	86	14	71	15
C57BL × BALB/c	38	16	24	100	19			18	23

*F1-hybrids were sired by the second indicated strain.

in the F1 hybrid BALB/c × C3Hf in the low-MuMTV class, only 33% of the animals develop a tumour at the relatively late age of 20 months, whereas in the high-MuMTV class, 86% of the animals contract the disease at the relatively early age of 14 months.

This picture becomes even more clear when tumour incidences and tumour ages are studied in the progeny of several crosses in relation to the MuMTV status at the first lactation period (Table 6). In every cross, tumour incidence is lower and tumour age higher in the so-called virus-negative groups. These groups with less than 5 μg of MuMTV antigens per 1 mg protein at first lactation also develop fewer tumours and at a later age than do the corresponding high-virus groups.

Especially in the first five crosses, which involve the GRS mouse strain, there is a striking difference in tumour age between the two groups with different amounts of MuMTV proteins in their milk. In 4 of these crosses, final tumour incidences are extremely high (more than 70%). It is noteworthy that the BALB/c mouse strain is involved in all of these cases.

DISCUSSION

The theory of genetic transmission of murine mammary tumour viruses originated in the purported single-genic control gamete-borne transmission of a virulent MuMTV in the GRS mouse strain [1–5]. The conclusion was based mainly on the incidence of palpable mammary tumours within a certain time period in a great variety of crosses involving the GRS mouse strain. By use of a rapid standardized tumour induction technique and an immunoassay for MuMTV antigens, van Nie and collaborators [12–14] concluded that indeed a single dominant gene of the GRS strain would cause release of large amounts of virus and early tumour development.

The theory has now been widely accepted, but its original basis—the single GRS gene associated with MuMTV transmission—has been seriously challenged [17–19]. In one study [18], the sensitive radioimmunoassay was used for the detection of MuMTV proteins in the milk. We employed the comparatively sensitive Sepharose bead immunofluorescence assay, the advantages of which have been discussed before [22]. After repeated absorption with nonviral materials, the antiserum proved to be sufficiently specific with this technique. No reactions were found with Rauscher murine leukaemia virus, normal BALB/c mouse serum or an extract of BALB/c lactating mammary gland. Specificity of the technique can also be concluded from the negative reactions of the C57BL and BALB/c milk samples. These strains are generally regarded to be negative for MuMTV in their milk. The technique is also sufficiently sensitive: the lowest detectable amount of MuMTV being 5 ng per sample of 50 μl.

In the crosses involving a GRS-hybrid male, there is a chance for nonchromosomal male transmission. We have therefore chosen a single mating of a female with such a male. It seemed that only one of the

Table 6. Mammary tumour incidences of progeny of several crosses in relation to MuMTV status

Cross No.	Female	F1-hybrid male	Number of animals	MuMTV-negative Tumour %	MuMTV-negative Tumour age (months)	<5 μg MuMTV Tumour %	<5 μg MuMTV Tumour age (months)	>5 μg MuMTV Tumour %	>5 μg MuMTV Tumour age (months)	Total Tumour %	Total Tumour age (months)
1	C57BL	C57BL × GRS	100	9	16	100	15	100	8	55	9
2	C57BL	BALB/c × GRS	48	35	19	50	19	96	8	71	11
3	BALB/c	BALB/c × GRS	103	20	21	74	20	100	7	78	12
4	BALB/c	C57BL × GRS	135	50	22	67	19	100	7	75	12
5	BALB/c	C3Hf × GRS	192	36	24	50	21	91	10	96	11
6	BALB/c	BALB/c × C3Hf	277	14	21	20	20	44	15	26	17
7	C3Hf	BALB/c × C3Hf	104	11	23	33	19	60	17	54	17
8	C3Hf	C57BL × C3Hf	111	14	21	16	22	39	18	26	20
9	C57BL × C3Hf	C57BL × C3Hf	68	20	23	0		69	20	34	22
10	BALB/c	C57BL × C3Hf	84	17	20	50	17	73	14	31	18

C57BL females became infected. She subsequently transferred the virus via the milk to her offspring. Approximately 10% of the BALB/c females were infected by their GRS-hybrid mates. In the studies of Heston *et al.* [18, 19], neither milk-borne transmission nor extrachromosomal male transmission of MuMTV seemed to be controlled as in our study. This may partly account for the aberrant ratios of MuMTV-positives and tumour incidences, which do not seem to fit a single-gene postulate.

In our study in three crosses involving the GRS strain, incidences of MuMTV-positive milk samples which are close to 50%, as would be expected in case of a single gene were found. Van Nie *et al.* [12–14] have also tested second back cross generations and the results were consistent with the one-dominant gene postulate. They called this gene "Mtv-2".

However, in the BALB/c × (BALB/c × GRS)F1, the incidence of MuMTV-positive milk samples is greatly in excess of 50%. Extrachromosomal male transmission of the virus may account for a maximum of 10% of this excess, but probably less, as there is a good chance that progeny mice receive the virus directly from their father in a genetic way as well as indirectly via the milk from their infected mother.

The excess in BALB/c × (BALB/c × GRS)F1 can only be explained on the basis of more than one gene controlling release of a MuMTV. The virus-positive milk samples have been arbitrarily divided into two groups with 5 μg MuMTV protein per mg milk protein as a cut-off point. In the group above 5 μg, most animals contain excessively large amounts of viral antigens in their milk (82 μg ± 5). Tumours appear extremely early (7 months average) in this group. In the other group, a high tumour incidence (74%) is found, but tumours appear late (20 months). We conclude from this that, in this cross, a single dominant gene segregates, which causes release of large amounts of virus and early tumour development in approximately 50% of the back cross animals. In addition, a second gene which is associated with the release of small amounts of viral antigens and late tumour development manifests itself in this cross. This probably represents a virus other than the virulent MuMTV-P, causing pregnancy-dependent tumours.

Nandi and Helmich [17] made the reciprocal cross and by an elegant procedure

excluded virus transmission via the milk. From the incidences of mammary lesions (neoplasms and hyperplastic nodules) in the offspring before 1 yr of age, they concluded that two independently segregating dominant genes in the GRS strain would control the transmission of a MuMTV, or at least the production of mammary lesions.

We do find two distinct populations in the backcross of the GRS-hybrid to BALB/c: one developing tumours at an early age (7 months) and one producing mammary carcinomas at the average age of 20 months. In the study of Nandi and Helmich, the F1-hybrids of BALB/c and GRS have a 100% tumour incidence at 7 months of age. They found 75% at that age in the F2 generation. This is in agreement with a single-gene hypothesis; in the case of a two-gene hypothesis, a 94% incidence would be expected. In the back cross to BALB/c, they found a 53% incidence at 7 months, which is in accordance with the single-gene postulate; otherwise, 75% would be anticipated. At 12 months of age, they found a considerably higher incidence of mammary lesions. This may be partly due to the second gene, postulated by Van Nie *et al.* [14] and by us, which is associated with a lesser oncogenic virus. This MuMTV-strain might induce hyperplastic nodules at one year of age which will develop into tumours after a considerable length of time, like the nodules induced by MuMTV-L of the C3Hf mouse strain [32].

In the four first crosses involving the GRS strain (Table 3), the proportion of mice with large amounts of MuMTV antigens in their milk is close to 50%. The great majority of these mice develop tumours before one year of age. This finding supports the notion of a single dominant gene which causes the release of a great amount of virus and early tumour development in the GRS strain. The relatively high proportion of mice with small amounts of MuMTV antigens in the milk, as found in the back cross to BALB/c (cross No. 3), is not encountered in the four other crosses involving the GRS strain. Probably the BALB/c strain carries recessive genes which allow the expression of the second GRS mouse strain gene for another MuMTV.

The excess over 50% of MuMTV-positives (also in the high-virus category) in cross No. 5, BALB/c × (C3Hf × GRS)F1, can be explained by expression of the MuMTV-L of the C3Hf strain. In the latter strain, viral antigens are released in considerable amounts, although not as much as in the GRS strain. The presence of mice which only release MuMTV-L in the so-called high-virus category explains very high tumour incidence (91%) as well as the increased average tumour age (10 months). Since the incidence of virus-positives in this cross is not 100% as compared to the F1-hybrids of BALB/c with either C3Hf or GRS, it must be concluded that the GRS-gene controlling release of virulent MuMTV-P is not allelic to the C3Hf gene controlling release of MuMTV-L. No conclusion can be drawn about allelism between the second GRS gene controlling release of a lesser oncogenic MuMTV-strain and the C3Hf gene.

From the back crosses of (BALB/c × C3Hf)F1 to either parental strain, it can be concluded that a single dominant gene of the C3Hf strain causes release of MuMTV. This has been found earlier by Van Nie and Verstraeten [15], who called this gene "Mtv-1". The BALB/c strain, which does not release a MuMTV into the milk at an early age, has a similar tumour incidence and tumour age as the virus expressor C3Hf. In the F1-hybrids of these strains, tumours appear somewhat earlier and in a considerably higher incidence. This indicates the BALB/c strain to be rather susceptible to MuMTV-L, as was also found in bioassays (Table 1). In both back crosses, tumour development is enhanced and accelerated when large amounts of virus are released.

In the F1-hybrids of C57BL and C3Hf, expression of MuMTV is largely inhibited. The results of the back cross to C3Hf suggest that a single dominant C57BL gene inhibits the release of MuMTV-L to a large extent. From cross 10, BALB/c × (C57BL × C3Hf)F1, it can be concluded that the C57BL gene inhibiting MuMTV release segregates independently from the C3Hf gene "Mtv-1" which allows the production of MuMTV-L; otherwise, a 50% incidence of virus-positives would be expected. It must be assumed that the C57BL gene, which we propose to call "Imv" (Inhibitor of mammary tumour virus release), is epistatic to "Mtv-1". The latter gene cannot function in its presence. Possibly "Imv" also inhibits the expression of the second GRS strain gene which is associated with the release of small amounts of MuMTV.

Surprisingly, the hybrid (C57BL × C3Hf)F1 produces mammary tumours in approximately the same incidence as does the parental C3Hf, despite inhibition of MuMTV-expression at an early age. However, tumours appear at a considerably later age. In all crosses involving this hybrid (Nos. 8–10), there is some correlation between the presence of viral antigens in the milk at a young age and high tumour incidences and relatively young tumour age, but it is still noteworthy that a substantial number of tumours are produced in the so-called virus-negative categories. This also holds for several other virus-free groups in Tables 5 and 6.

It is known that the endogenous MuMTV-O is switched on at a late age in the BALB/c strain [26, 33]. Infectious virus can be recovered from 9% of spontaneous BALB/c mammary tumours [33] and small amounts of MuMTV antigens can be found in the majority of BALB/c tumours [15]. It is possible that, for instance, inhibition by the "Imv" gene breaks down on ageing in (C57BL × C3Hf)F1 leading to virus release and subsequent tumour production. An alternative possibility is the expression of only the gene for oncogenic transformation, called "mam", of the endogenous MuMTV. It must be kept in mind, however, that mammary tumours may develop without any involvement of an endogenous virus-gene.

Molecular hybridization studies indicated that several mouse strains contain multiple copies of the MuMTV genome in their normal cellular DNA [8–10]. The GRS strain contains an exceedingly high number of such copies [9–10]. We assume the "Mtv-2" gene to be associated with this excess in MuMTV proviral genomes; in other words that they are located in tandem at a single locus as has been postulated for RD114 and the protovirus for feline leukaemia virus in the domestic cat [34]. This cluster of proviral genomes is continuously transcribed without interference of any other cellular gene, giving rise to high virus titers and early tumour development.

The "Mtv-1" gene of the C3Hf allows continuous release of MuMTV-L, but does not induce the early appearance of the virulent MuMTV-O harboured by the BALB/c genome in the F1-hybrid with BALB/c. Reciprocally, BALB/c strain genes inhibit the expression of any of their own proviruses, but not affect the production of the endogenous MuMTV-L from C3Hf. We interpret these findings to mean that the germinal proviruses of MuMTV are controlled by closely linked regulatory genes. Only when in *cis*-position can they repress the neighbouring provirus. Such genes have no effect on proviruses at other sites in the mouse genome. In the C3Hf, such a gene would be mutated, giving rise to early virus release.

The "Imv" gene of the C57BL is epistatic to the "Mtv-1" gene of C3Hf and the gene(s) in the GRS are associated with a lesser oncogenic virus. It inhibits the expression of these viruses to a large extent. Position effect does not seem to play a role in the action of that gene; although not linked to "Mtv-1", it can interfere with its action. The "Imv" resembles the "I^e" gene in chickens [35], which is epistatic to the gene(s) allowing expression of group-specific antigens by the endogenous RAV-O virus. It remains to be investigated, however, whether the action of the "Imv" gene would be interference with the establishment of a somatic provirus.

Several mouse strains, derived from high-mammary cancer strains after removal of milk borne MuMTV-S spontaneously release a low-oncogenic MuMTV [36]. Probably the genes like "Mtv-1" allowing release of an endogenous virus also cause susceptibility to superinfection with MuMTV-S. It would be interesting to know whether such genes in other mouse strains are allelic to "Mtv-1".

The expression of low-oncogenic MuMTV in the other mouse strains is also inhibited by the C57BL genome in the respective F1-hybrids, most likely due to the "Imv" gene. Several other nonexpressor strains such as O20 and CBA inhibit the expression of MuMTV in F1-hybrids with C3Hf. It would be of interest to know whether the genes responsible for this phenomenon are allelic to "Imv" of the C57BL.

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E.O.R.T.C. News

Study of *Cis*-Diammine-Dichloro-Platinum in Advanced Soft Tissue Sarcoma*†

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1. BACKGROUND AND INTRODUCTION

PROTOCOL 62761 of the Soft Tissue Cooperative Group is presently studying 2 combination chemotherapy regimens consisting of adriamycin, DTIC, cyclophosphamide and vincristine in patients with advanced soft tissue sarcoma. In the first arm (S_1) the four drugs are given simultaneously on day 1, while DTIC is continued during 5 days. In the second arm (S_2) adriamycin and DTIC are given simultaneously without cyclophosphamide and vincristine. The latter 2 drugs are alternated with the former 2 drugs every 4 weeks. The most active drugs in the treatment of soft tissue sarcomas are adriamycin and DTIC. The combination of these 2 drugs has proven to be more effective in soft tissue sarcoma than each of the 2 drugs given as a single agent. Vincristine and cyclophosphamide are quite ineffective in soft tissue sarcomas when given as single agents. Recently, *cis*-diammine-dichloro-platinum (*cis*-DDP) has proven to be effective in several solid tumors such as testicular carcinoma, ovarian cancer, bladder cancer and possibly in head and neck tumors as well [1–4]. The only data available on the effect of *cis*-DDP in soft tissue sarcoma consist of 5 available patients in a phase I study [2]. No response was observed in these patients. The Soft Tissue Sarcoma Group offers an excellent patient population to perform a phase II trial of *cis*-DDP in soft tissue sarcoma. This phase II study can be performed in patients who go off study in the protocol for advanced sarcoma (62761) and will give us information on the response rate of this tumor within 6 months from now. In case *cis*-DDP would prove to be active in soft tissue sarcoma our next step will be to initiate a protocol in advanced sarcoma including this drug in combination regimens. As all the investigators participating in the study for advanced soft tissue sarcoma are internists we feel that it is justified to introduce this toxic drug into the Soft Tissue Sarcoma Cooperative Group.

2. PURPOSE OF THE STUDY

(a) The main purpose will be to determine objective tumor response rate and duration of response to DDP in patients with advanced soft tissue sarcoma who have failed all treatment presently available.

(b) To determine the morbidity of *cis*-DDP treatment in this group of patients.

3. SELECTION OF PATIENTS

3.1 *Conditions for patient eligibility*

Patients with advanced soft tissue sarcoma who have failed on conventional treatment. This includes patients leaving protocols 62761 and 62771 because of progression and patients

*E.O.R.T.C. Soft Tissue Sarcoma Cooperative Group.

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who are not eligible to enter 62761. The patient should have a measurable lesion with progression during the 4 weeks prior to treatment.

3.2 *Conditions for patient exclusion*

Patients 20 yr old or younger, patients above 70 yr and patients with abnormal kidney function: creatinine clearance below 80! Unfavourable, haematologic status; WBC below 3000/mm^3 and platelet count below 150,000/mm^3, cytostatic agents during the previous four weeks, performance status according to Karnofski under 50, central nervous system lesions, intercurrent infection and overt psychosis or marked senility.

4. DESIGN OF THE TRIAL

All patients will receive the same treatment schedule consisting of DDP in dextrose 5% infusion containing mannitol. The infusion will be given over 3 hr. If during the 1st course 100 mg/m^2 DDP is tolerated without toxicity, the dose will be increased to 120 mg/m^2 in the subsequent courses. This treatment will be repeated every 3 weeks. The response will be evaluated after the second course. If at 6 weeks there is tumor regression or no change, treatment will be continued until progression. If there is progression at 6 weeks the patient will go off study. Patients responding to treatment must be followed to progression and to death whenever possible.

5. THERAPEUTIC REGIMENS AND TOXICITY

5.1

Prior to the DDP infusion the patient should receive 1 l. of saline over a period of 4 hr. *Cis*-DDP 100 mg/m^2 will then be given in 1 l. dextrose 5% containing 37.5 g of mannitol over a 3 hr period. Diuresis should be maintained by giving an additional 3-liter saline infusion over the following 24 hr. In case the urine output is insufficient (<600 cm^3 per 6 hr) furosemide 20–40 mg/day should be added to the regimen. Thus, careful monitoring the urine output is essential! This treatment will be repeated every 3 weeks. If during the 1st course 100 mg/m^2 DDP is tolerated without toxicity the dose of DDP will be increased to 120 mg/m^2 in the subsequent course. The patient will be evaluated after 2 courses. The treatment will be continued until progression in case of no change or regression after 6 weeks. In case of progression at 6 weeks the patient will go off study.

5.2 *DDP toxicity*

The most important toxicity is renal failure. However, a good renal function prior to treatment and an adequate urine output during the 24 hr of treatment makes this complication unlikely [2, 5].

Auditory abnormalities consisting of a high pitch hearing loss may occur. Myelosuppression, reaching its nadir at day 10–14, is often seen but is not severe. Anaphylactic reactions are rare. Antiemetics should be given but are only slightly effective. In case of infectious complications following the treatment, gentamycin should be avoided whenever possible as there is probably a cumulative toxicity to the kidney. The same is true for damage which may be caused to the auditory nerve by gentamycin. In case gentamycin has to be given, never add cephalothin to this regimen as this combination is still more toxic to the kidney. Serious nephrotoxicity has been observed with this combination [6]. In case the serum creatinine increases during treatment to a level above 1.2 mg/100 ml the kidney function will, in most cases recover within 3 weeks. If, however, at 3 weeks the serum creatinine is still above 1.2 mg/100 ml the next dose should be postponed for a maximum of one week (4 weeks after previous dose). If at 3 weeks after the previous course the WBC count is below 3000 or the platelet count is below 150,000 the treatment should be postponed as well for 1 week. In case treatment has to be postponed for more than 1 week the patient should go off study due to toxicity.

6. REQUIRED CLINICAL EVALUATION, LABORATORY TESTS AND FOLLOW-UP

6.1

Age, sex, case history and duration of symptoms (specify), date of clinical and pathological diagnosis and how this was established, prior treatment (data and methods). The physical examination should include: height and weight, the Karnofski index and exact description and measurement of the measurable parameters.

6.2

The minimal laboratory studies required are: complete bloodcount creatinine clearance, liver function tests consisting of bilirubine, alkaline phosphatase, gammaglutamyl *trans*-peptidase, SGOT, SGPT and if possible cholinesterase. Furthermore, urinanalysis should be done. Because of the possible ototoxicity an audiogram should be performed prior to treatment as well. Radiological examination should include a chest X-ray and a skeletal survey. An isotopic bone scan is optional.

6.3

Follow-up. Before each treatment course the morbidity of the previous course should be registered. Furthermore, a clinical examination, blood investigations, and evaluation of the Karnofski index are required. An audiogram will be repeated after the second course. Any unexpected side effects of treatment should be reported immediately to the study coordinator. A clear description of the tumor response is required prior to the third course.

7. CRITERIA OF EVALUATION

Patients are considered evaluable if they have been treated with a minimum of 2 courses.

7.1 *Complete remission*

Disappearance of all symptoms and signs of soft tissue sarcoma for a minimum of 3 weeks.

7.2 *Partial remission*

Significant decrease in size in at least 50% of all lesions for a minimum of 3 weeks while the remainder are static. Such a decrease is defined as a reduction by 50% of the product of the two largest perpendicular tumor diameters. Changes in body chemistry and improvement in hemopoiesis cannot be construed as criteria of objective remission.

7.3 *Stable disease*

Less than 50% reduction in the product of largest perpendicular diameters of measurable lesions or less than 25% increase in any of the measurable lesions. No appearance of any new lesion.

7.4 *Progression*

Increase of more than 25% in the product of the largest perpendicular diameters of any lesion. Also, appearance of any new lesions regardless of the response of other lesions.

7.5 *Early death*

Death occurring during the first three weeks due either to tumor progression or drug toxicity.

8. REGISTRATION

A patient is registered after the local pathology review by telephoning to the E.O.R.T.C. Data Center, phone: Brussels: 538.65.33 or by telex 22.773 from 9.00 a.m. to 5.00 p.m., Monday–Friday. The following information is requested:

1. protocol number (62781),
2. name of the institution,
3. name of the patient,
4. histological type,
5. name of the responsible investigator.

9. SUBMISSION OF FORMS

All forms are to be sent in duplicate to the E.O.R.T.C. Data Center, Institut Jules Bordet, 1, Rue Héger Bordet, 1000 Bruxelles, Belgium.

The schedule for submission of forms is as follows:

1. *On-Study form*

Send within one week of the patient's entry on study, 1 page.

2. *Chemotherapy form*

Send after completion of each 2 cycles of DDP, 1 page.

3. *Flow-sheet*

The first flow sheet should be filled out prior to the first cycle of chemotherapy and sent with On-Study form. It should also be filled out at 6 weeks and thereafter every 12 weeks as long as the patient remains on-study. Also immediately upon progression, 1 page.

4. *Measurement form*

Send with on-study, at 6 weeks and every 3 weeks thereafter as long as the patient remains on-study. Also immediately upon progression.

5. *Summary form*

To be sent upon progression or death, whichever comes first.

10. STATISTICAL CONSIDERATIONS

The lowest limit of therapeutic activity considered to be of interest is a response rate of 20%. Twenty-nine patients will be entered on study and the drug rejected from further study if 3 or fewer responses are obtained. If no response have been obtained in the first 19 patients treated the study will be stopped. This plan insures that if the drug has a response rate of at least 25% the probability of rejecting the drug from further study is approximately 0.05.

11. ADMINISTRATIVE RESPONSIBILITY

This study is a joint effort among the investigators working in the E.O.R.T.C. Soft Tissue Sarcoma Cooperative Group. The secretary of the group will evaluate the clinical data of patients while the statistical analysis will be performed by Dr. Richard Sylvester. Dr. Sylvester can be reached at the Data Center.

The address of the Secretary Dr. H. M. Pinedo is: Oncology Unit, Department of Internal Medicine, University Hospital, Utrecht, The Netherlands. Telephone: 030-372202.

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Letters to the Editor

The Relationship Between Inflammatory Responses and WBP1 Tumour Cell Attachment to the Rat Omentum

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WHILE much attention has been focussed on the inherent properties of neoplastic cells which confer invasiveness, the influences exerted on the process by the host have remained more obscure [1]. A close relationship between inflammatory responses and invasion by both normal cells (during pathological and morphogenetic processes) and neoplastic cells has been previously suggested [2]. During invasion of murine abdominal organs by ascites tumour cells certain regions of the viscera were invaded earlier than others and tumour cell adhesion and infiltration appeared to follow host responses to the tumour [3, 4]. Attachment to the abdominal wall appeared to be preceded by the breaking of mutual contact by the mesothelial cells and their exfoliation, resulting in the rapid adherence of tumour cells to the exposed membrane [5].

As a result of studies on the dissemination of ^{125}I-iodo-deoxyuridine labelled cells of the sublines of different malignancy of the WBP1 ascites tumour [6] after i.p. injection into black and white hooded rats, which demonstrated that more cells of each subline attached to the omentum than to any other organ or tissue, we undertook a study of the process in the light and scanning electron microscopes. Evidence is presented of the close link between inflammatory response and tumour cell attachment to the omentum.

Omenta were removed from groups of animals either without treatment or at intervals after i.p. injection of either 5×10^7 WBP1 cells in Tyrodes–gelatin–citrate medium or medium alone. The omenta were prepared either for observation in the SEM as described previously [7] with diffusion dehydration in acetone followed by critical point drying, or for histological examination by fixation in 10% formal saline containing 1% calcium chloride and staining 4 μm sections with haematoxylin and eosin.

By 8 hr after injection of tumour cells there was evidence in the SEM of a reaction in the surface of the taches laiteuses (Fig. 1) which did not occur after injection of medium alone. The smooth-surfaced mesothelial cells in many areas appeared to be breaking mutual contact, creating openings in the surface, and the number of cells attached to the surface was greater than normal. Many of the attached cells could have been WBP1 cells, having a diameter of 6 μm and a surface comprising closely packed microvilli, although some peritoneal cells from normal animals were similar in appearance. The mesothelium overlying adjacent adipose tissue appeared normal, with no evidence of cell attachment.

The appearance of the surface of the taches laiteuses then changed progressively, until at 24 hr after tumour injection these areas contrasted markedly with the mesothelium overlying adjacent adipose tissue (Fig. 2). The surface of the taches laiteuses comprised a mass of cells *ca.* 10 μm in diameter and smaller cells characteristic of WBP1 cells so that in many areas the mesothelium appeared to be entirely disrupted, whereas the mesothelium overlying surrounding adipose appeared normal, with no evidence of cell attachment.

Histological examination of omenta 24 hr after injection of tumour confirmed that characteristic WBP1 cells [8] were present at the surface of and within the taches laiteuses at

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this time. The mesothelium in these areas was difficult to recognise and there was evidence of an inflammatory response with an influx of polymorphonuclearcytes and some mast cells. Elsewhere the tissue appeared normal. After this time it was impossible to recognise the mesothelial surface of the taches laiteuses, tumour cells increased in evidence and the intensity of the inflammatory response increased progressively, with the appearance of large foci of the histiocytic cells characteristic of normal taches laiteuses. There was evidence of invasion of adipose tissue adjacent to some taches laiteuses by isolated WBP1 cells, until at 72 hr after tumour injection considerable invasion of adipose tissue had occurred. Even at this time the mesothelium overlying invaded and other adipose tissue still appeared normal with no evidence of tumour cell attachment.

We have clearly illustrated that tumour cell attachment is closely linked to the host inflammatory response. The breaking of mutual contact by the mesothelial cells created openings in the surface, perhaps exposing a more suitable surface for tumour cell attachment. That such a reaction was a prelude to attachment was strongly suggested by the fact that as the omentum is covered by the same continuous mesothelium there was no reason if this was not the case why tumour cells should not have attached to sites other than the taches laiteuses. The ensuing intense inflammatory response appeared to offer ideal opportunity for the establishment of tumour foci, and led to subsequent invasion of surrounding tissue. The inflammatory response, previously shown to cause differences in tumour cell attachment to various organs [3, 4] has in this case been shown to cause differences in cell attachment to different regions of the same organ.

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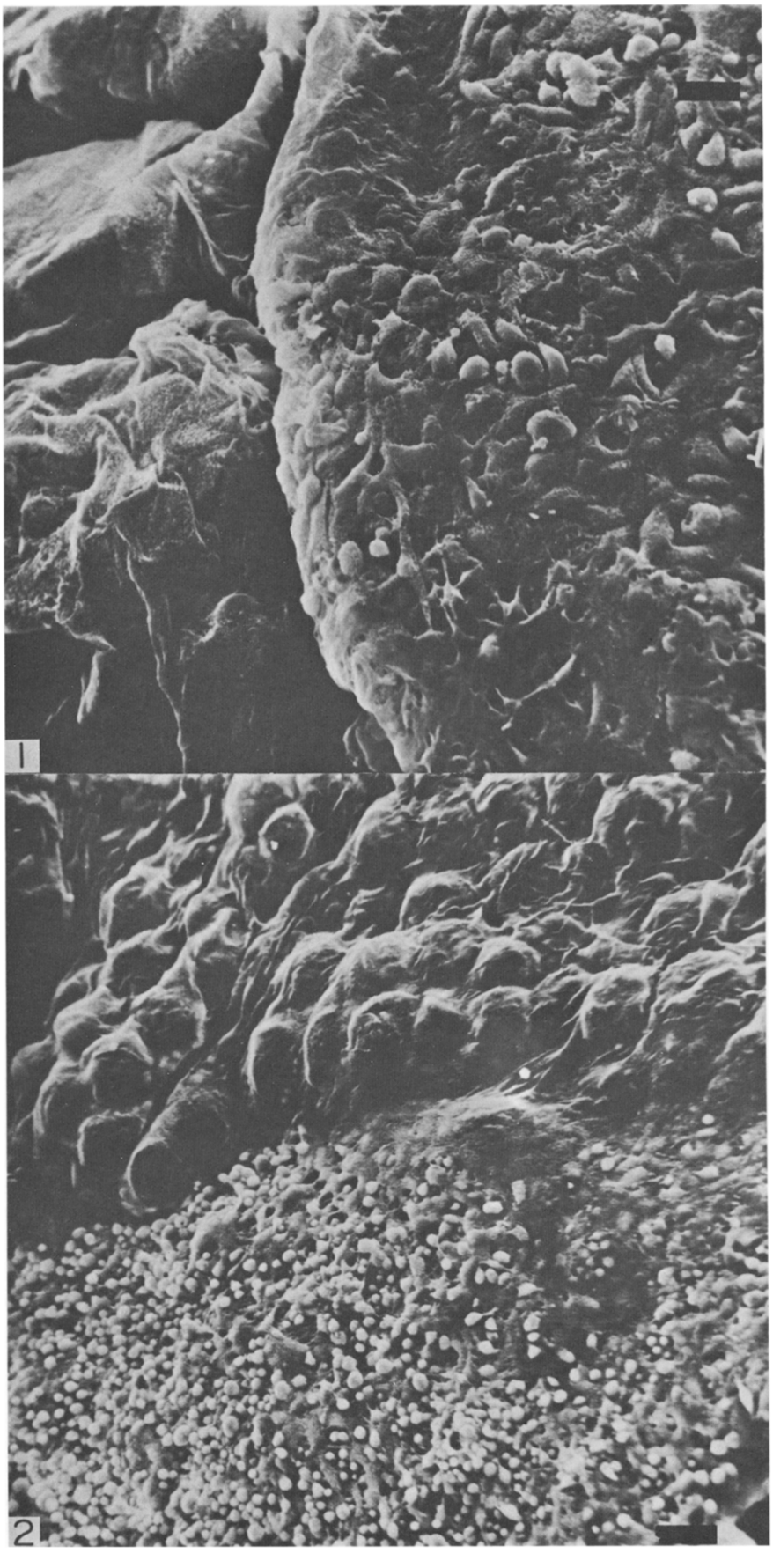

Fig. 1. *Scanning electron micrograph of the rat omentum* 8 hr *after i.p. injection of* 5×10^7 *WBP*1 (*X*) *cells. Scattered cells are attached to the surface of a tache laiteuse* (*left*), *the mesothelial cells of which appear in some areas to be breaking contact creating openings in the surface. The mesothelium overlying adjacent adipose tissue appears normal. Bar represents* 20 μm.

Fig. 2. *Scanning electron micrograph of the rat omentum* 24 hr *after i.p. injection of* 5×10^7 *WBP*1 (*X*) *cells. Many cells are attached to the surface of a tache laiteuse* (*bottom*) *in contrast to the mesothelium overlying surrounding adipose tissue, to which there was no evidence of cell attachment. Bar represents* 100 μm.

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Letters to the Editor

Acute Non-Lymphoblastic Leukemia

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THE MOST recent reports [1, 2] of two controlled clinical trials of chemoprophylaxis in early breast cancer have confirmed that adjuvant chemotherapy to radical mastectomy in primary breast carcinoma with positive axillary lymph nodes can improve the 3 yr relapse rate. Both single agent [3] and combination chemotherapy seem to be in general well tolerated, without any long term side effects such as drug related second malignancy [4]. Notwithstanding these successful results, many physicians have recommended caution since this approach should be still considered experimental [5–8]. The potential risk of a second drug-induced neoplasia is always present because alkylating agents, used in the above mentioned treatments, may be immunosuppressive and carcinogenic, facilitating the onset of a second malignancy.

This letter describes a case of acute promyelocytic leukemia arisen after prolonged adjuvant treatment with L-PAM, after surgery, for breast cancer.

Case report. A 39-yr-old premenopausal woman, who undervent radical mastectomy in February 1976 for a carcinoma of the right breast (T_2 N_{1b} M_0, estrogen and progesterone receptor negative) has been treated on out-patient basis with adjuvant chemotherapy (L-PAM: 0.15 mg/kg p.o. × 5 every six weeks for a total dose of 400mg) from March 1976 to January 1977 according to the protocol of Fisher *et al.* (1975) [3]. The woman, who stopped treatment with L-PAM in February 1977 because of severe leukothrombocytopenia (WBC 1800/mm^3, platelets 66,000/mm^3), was hospitalized in March 1977 for increasing anemia with fever and skin rushes. She appeared weak and pale, with petechiae over the arms, legs and abdomen.

A macular rush over the cheeks and left periorbital haematoma were also present. At the admission the blood picture was: HT: 20%, WBC: 10,900/mm^3 with a differential of 10% segmented neutrophils, 2% eosinophils, 0% basophils, 4% lymphocytes, 84% blasts. Platelets counts 120,000/mm^3. Two days later WBC rose to 14,000/mm^3 and platelets were 115,000/mm^3. Bone marrow aspirate was performed and marrow smears showed a differential of 78% blasts, 2% myelocytes, 2% metamyelocytes, 1% bands, 5% segmented neutrophils, 3% lymphocytes, 2% mature plasma cells, 3% basophilic normoblasts, 2% polichromatophilic normoblasts, 2% ortochromatic normoblasts. Cytochemical staining (PAS, peroxidase, naphtolASD-chloro-esterase, alpha-naphtil-esterase, naphtol-ASD-acetate-esterase with and without NAF) demonstrated both in peripheral blood and in bone marrow, blasts with marked positive reactions; a small percentage of these blasts were also blue toluidine positive. The coagulation parameters were: Plasma fibrinogen 0.03 g/ml (n. 0.20–0.40), fibrinogen degradation products (FDP) 84 μg (n. ≷ 8 μg), prothrombin activity 40% (n. 80–100%), plasma prothrombin time (Quick) 21 sec (n. 13–15 sec), plasma coagulation time (Howell) 5 min 05 sec (n. 1 min 30 sec–2 min 15 sec), partial thromoboplastin time (PTT) 55 sec (n. 35–45 sec). These clinical and laboratory findings allowed us to diagnose an acute promyelocytic leukemia with disseminated intravascular coagulation (DIC) [9–11]. Heparine and daunorubicine (40 mg/m^2 i.v.) were given for two days; unfortunately the woman died from massive

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cerebral haemorrhage a few days later. Permission for autopsy was not granted.

In our knowledge this is the first reported case of acute leukemia arisen after long-term adjuvant treatment with L-PAM for primary operable breast cancer. Obviously this case needs to be further confirmed and its incidence compared with the inherent susceptibility to development of leukemia in patients with breast cancer without treatment. Many papers [12–14] have described cases of acute leukemias after long-standing treatment with L-PAM for malignant (myeloma) and not malignant diseases. These leukemias have been variously classified : erithroleukemia, stem cell, myeloblastic, acute lymphatic, myelomonocytic and, in a few cases, promyelocitic leukemia.

From the haematological point of view, this report is interesting for the uncomon cytological type of leukemia induced by L-PAM. Moreover this observation cannot be neglected since it supports the fears of these clinicians [5–8] who have been warning the potential oncogenic risk of adjuvant chemotherapy. Our observation underline at least the importance of statements of Costanza [5] and British Breast Group [7] that chemoprophylaxis should not be considered a "standard procedure" and should not be used "electively in primary operable disease" until the ultimate value and drug related risk will be well known.

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Heat Sensitivity of Rat Skin

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Abstract—*Experiments were conducted to determine the heat sensitivity of normal tissues. The rat skin was selected as a suitable model. After inflation of the rat subcutis with air, anaesthetized animals were placed in a heated water bath with the upper part of this air pouch exposed to the hot water. A total of* 502 *animals were treated with temperatures ranging from* 42 *to* 47°C *and exposure times of from* 12 *to* 240 min. *The intensity of damage was divided into* 6 *grades ranging from "no effect" to "maximum effect". The relationship between the exposure time to heat and the required temperature for isoeffect damage was determined and expressed as* $t = a \times b^T$, *with a value of* 0.51 *for* b(t = *exposure time;* T = *temperatures;* a = *constant). The synergistic effect of heat combined with X-irradiation was also studied. When animals were X-irradiated within* 6 hr *after heat treatment, the synergistic effect was evident, but disappeared after* 24 hr.

INTRODUCTION

INTEREST in the use of hyperthermia for cancer therapy has increased. Hyperthermic treatment has been performed and analysed experimentally as well as clinically and its enhanced effects when combined with irradiation and drugs have also been reported (for review, see [1–3]). Hyperthermia will very likely be applied as one of the methods of cancer treatment if some physical problems can be solved [4].

Damage to normal tissues cannot be neglected when hyperthermia is applied in cancer. Although effects on skin have been reported in the combination of irradiation and hyperthermia [5–7], analyses of the effect of hyperthermia alone are few. In this report, rat skin was selected as a suitable model and was treated hyperthermally with temperatures of from 42 to 47°C and with exposure times of from 12 to 240 min. The hyperthermic effects were analysed quantitatively with the RIDIT system [8]. The synergism of hyperthermia and irradiation effects on rat skin was also investigated.

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MATERIALS AND METHODS

Six to eight-week-old WAG/Rij male rats (approx. 120 g) were used in this study. In order to expose only skin, the following method was adopted. Animals were anaesthetized with nembutal injected peritoneally (0.1 ml per animal). The hair on the back was removed by clipping and 30 ml of air was injected subcutaneously at the site to form an air pouch [9]. The air pouch was immersed in heated water. The i.p. injection of nembutal kept the animals anaesthetized for about 1 hr. For prolonged heat treatments, additional i.p. injections with nembutal were usually required. The animals were supported by a lucite "raft", with the air pouches extended into the water through rectangular 30 × 50 mm holes. The temperature of the water was controlled within 0.1°C. After exposure to heat, the air was removed from the pouch. A separate series of experiments were performed in order to measure the temperature of the skin during the treatment by implantation of a thermister (UUA 35J3, Omega Engineering Inc., Conn.).

Irradiation with X-rays was carried out after the hyperthermic treatment. The animals were anaesthetized only during irradiation and the hyperthermic treatment. The intervals between hyperthermia and irradiation ranged from 0 to 72 hr. Irradiation

was performed with a 300 kV Phillips Muller X-ray machine; HVL 3 mm Cu; exposure dose rate about 380 rad per min.

Ten animals were used for each treatment group. A total of 502 animals were treated; 18 of the animals died during the initial phase of the anaesthesia. No animals were lost due to the hyperthermic treatment, as the body temperature remained at 38–39°C. The animals were observed for signs of damage twice weekly by three persons. Clipped hair of non-treated animals was regrown in one week. The hyperthermic damage was manifested during the first week and the maximum amount of damage was observed at the 2nd and 3rd weeks. Slight symptoms of damage were delay of hair growth and epilation of the skin. More severe was induration of the skin and the maximum effect was necrosis. At 4 weeks after the treatment, some recovery from the damage was observed in the group showing slight symptoms. Epilation of the skin was fully recovered at 6 weeks after the treatment, but the recovery from induration of the skin was not observed even at 8 weeks. When animals were treated with a high temperature and a long treatment time, which eventually produced induration of the skin, erythema appeared in one day after the treatment and this changed to dark-brown colored dots which were due to intracutaneous hemorrhage in 1 week. At the same time, edema appeared. This skin showed induration at 2 weeks. Induration of the skin is, therefore, the final step of erythema, haemorrhage and edema. In animals showing the maximal response, i.e. heavy induration and/or breakdown of skin, the entire skin surface involved became a nonviable, brittle scale which sloughed off during the healing process resulting in scar formation. As the maximum symptoms appeared at the 3rd week and did not change appreciably over one week, two sets of observations in this week were used for the analysis. The intensity of damage was divided into 6 grades ranging from "A" (no effect) to "F" (maximum effect) as shown in Table 2.

Table 1. *The values of* a *and* b *in equation* (1)

Grades	a	b
B	3.12×10^{14}	0.51
C	9.52×10^{14}	0.50
D+E	3.51×10^{14}	0.51

RESULTS

Temperature of skin

The temperature of skin was determined after the exposure to water of 44°C; this is shown in Fig. 1. After 5 min, it was 43°C and

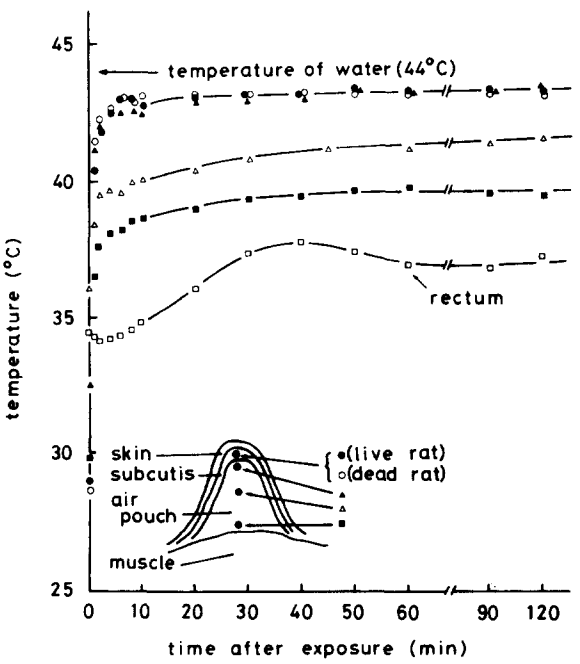

Fig. 1. *Temperature of the skin exposed to water of* 44°C. *The measured points are indicated on the cross-section of the air pouch.*

increased gradually to 43.3°C after 1 hr. As the increase in the temperature of the skin of dead animals showed the same pattern as that of live animals, the difference in temperature between the water and skin was due to the absorption of heat through the air of the pouch rather than blood flow. The temperature of the rectum reached 37°C in 30 min due to the absorption of heat, but the effects of heat on the body might be negligible.

Although the temperature of skin had increased to about 1°C below that of the water in 10 min, the exposure time was indicated from the time at which the air pouch was immersed in water and the temperature of treatment was taken as the temperature of the water in this work.

Relationship between temperature and exposure time

Animals were treated hyperthermally with water bath temperatures of 42–47°C with 1°C intervals and with exposure times of 12–240 min. In 50 observations of animals treated with 42°C for 240 min, 40 were scored as

grade "A", 9 as "B" and 1 as grade "C". The temperature of 42°C never produced damage greater than grade "C", even with the prolonged exposure time of 240 min. Total numbers of scores of the entire experiment were 383 as "A", 175 as "B", 154 as "C", 122 as "D", 109 as "E" and 511 as "F". These data were used for the determination of the relationship between temperature and exposure time for isoeffect. Obviously, the scores of the lower threshold, i.e. grade "A" (no effect) and the upper threshold, score "F" (maximum effect), cannot be used to obtain isoeffect lines. Therefore, only the "B", "C", "D" and "E" scores were used. As the frequencies of occurrence of symptoms of grades "D" and "E" were lower than those of other grades, the grades "D" and "E" were analysed as one grade. The relationship between the temperature and the required exposure time which produced damage of the same degree was obtained for grades "B", "C" and "D+E" and is shown in Fig. 2. The mean and its 95% confidence interval of exposure time producing each damage of grade are shown. The lines were drawn by the least squares method. The following relationship was observed between the temperature (T) and exposure time (t):

$$t = a \times b^{T} \qquad (1)$$

where a and b are constants. The value for a indicates the time in minutes at which the lines are extraporated to the temperature of 0°C and shows the position of the lines. The value for b indicates the slope of the lines. These values are shown in Table 1. The value for b was almost the same for three degrees of damage. The weighted mean value for b was 0.51.

Quantitative analysis of damages

Applying the RIDIT system designed by Bross [8], grades "A" to "F" were quantified according to the frequencies of occurrence of each symptom. In this system, the frequencies were used to derive a cumulative intensity scale from 0 to 1. The degrees of damage which are expressed by RIDIT are shown in Table 2.

The damages occurring hyperthermally are a function of both the temperature and the exposure time. As the treatment was performed with different temperatures and exposure times, all data were normalized to a 60-min exposure time by using the following equation derived from equation (1);

$$T = \frac{\log (60/t_0)}{\log b} + T_0 \qquad (2)$$

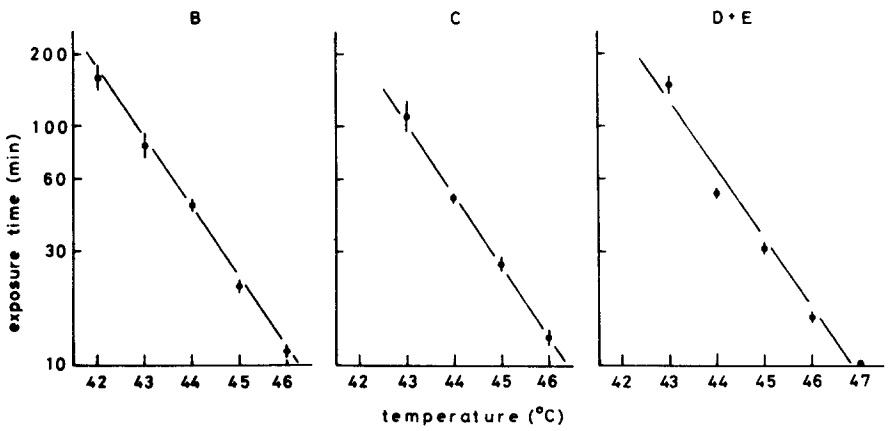

Fig. 2. Isoeffect curves for damage of grades "B", "C" and "D+E". The mean (solid circles) and its 95% confidence interval (vertical bars) of exposure time were calculated from the frequency of each damage at different temperatures.

where T_0 and t_0 are the temperature and exposure time, respectively, of one treatment condition to be normalized; T is an equivalent temperature of the condition for the 60-min exposure time. The results are shown in Fig. 3. The mean and its 90% confidence interval of temperature were obtained by average smoothing. Below 43°C, there was no damage; between 43 and 44°C, reparable damage occurred. When a temperature of more than 44°C was used, irreparable damage was produced.

To confirm the results shown in Fig. 3, additional experiments in which the intervals of temperature were less than 1°C were performed for a constant 60-min exposure. The

Table 2. Rat skin reaction after hyperthermic treatment

Grades	Symptoms	Degrees (RIDIT)
A. No effect	Normal skin	0.1317
B. Minimum effect	Delay of hair growth	0.3235
C. Slight effect	Slight epilation	0.4367
D. Obvious effect	Morderate epilation and/or slight induration	0.5316
E. Intense effect	Severe epilation and/or moderate induration	0.6110
F. Maximum effect	Heavy induration and/or break down of skin	0.8242

results are shown in Fig. 4. The curve in the figure is reproduced from Fig. 3. The experimental data are well fitted to the curve.

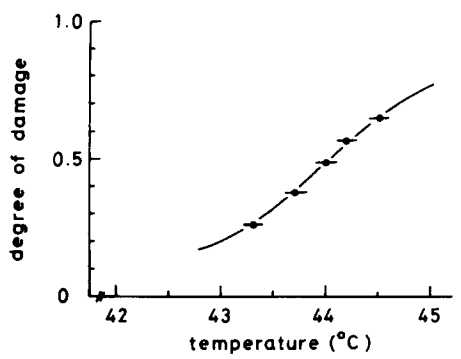

Fig. 3. Relationship of the degree of hyperthermic damage and equivalent temperature for a 60-min *treatment. The values for temperature were normalized to* 60 min *by means of equation* (2) *for all data and the mean and its* 95% *confidence interval of temperature were obtained by average smoothing.*

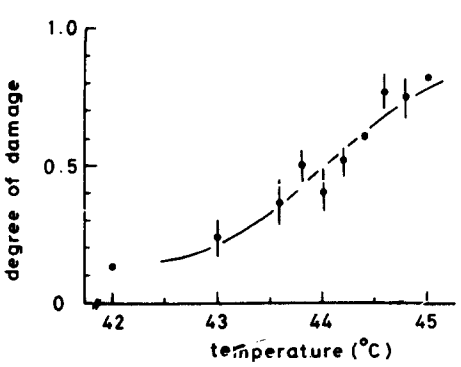

Fig. 4. Relationship of the degree of hyperthermic damage and indicated temperature for a 60-min *treatment time. The curve is from Fig. 3.*

This indicates that the relationship between the temperature and damage shown in Fig. 3 may be applied to any treatment within the range of 42–47°C when converted equivalently from one treatment schedule (T_1, t_1) to another treatment schedule (T_2, t_2) by using the following equations derived from equation (1):

$$T_2=\frac{\log\,(t_2/t_1)}{\log\,b}+T_1 \tag{3}$$

$$t_2=t_1 b^{(T_2-T_1)} \tag{4}$$

Hyperthermia combined with X-irradiation

Skin damage produced by hyperthermia alone or by a combination of hyperthermia and X-irradiation was scored at the 3rd week of treatment. Symptoms of X-irradiation and their RIDIT's are shown in Table 3. Figure 5 shows the degrees of damage produced by X-irradiation alone.

To determine the interval which produced or abolished a synergistic effect, animals were first treated hyperthermally at 44°C for 30 min, which corresponded to the treatment at 43°C for 60 min. This produced damage between grades "A" and "B" (Fig. 3). They were then X-irradiated with 2000 rad at different intervals. X-irradiation alone produced damage between grades "C" and "D" (Fig. 5). As additional hyperthermic treatment of such an exposure enhanced radiation-induced damage (data not shown), the damage produced by the combined treatments was analysed according to the symptoms shown in Table 3. The effect of combined treatment at different intervals is shown in Fig. 6. When X-irradiation was applied immediately after hyperthermic treatment, the maximum effect (moist desquamation of more than half of the treated area; grade "G") was observed. The damage was decreased with an increase in the interval between the treatments. This result agreed well with previous reports on tumor cells *in vivo* [6] and cultured cells [10], where the synergistic effect disappeared when the

Table 3. Rat skin reaction after X-irradiation

Grades	Symptoms	Degrees (RIDIT)
A. No effect	Normal skin	0.1135
B. Minimum effect	Delay of hair growth	0.2873
C. Slight effect	Slight and moderate epilation	0.4066
D. Moderate effect	Heavy epilation	0.5732
E. Obvious effect	Dry desquamation	0.7356
F. Intense effect	Moist desquamation (less than half area)	0.8606
G. Maximum effect	Moist desquamation (more than half area)	0.9655

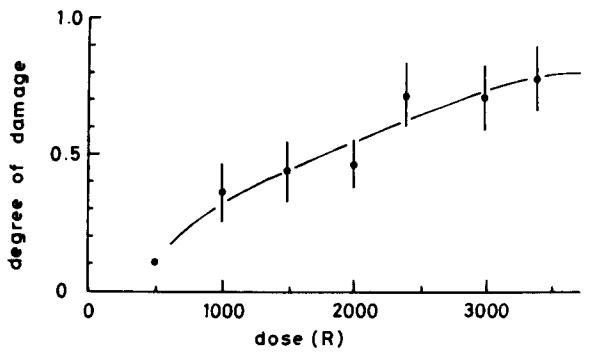

Fig. 5. Relationship of degree of damage by X-irradiation and irradiation dose. Grades of damage, "A" to "G", are indicated in Table 3.

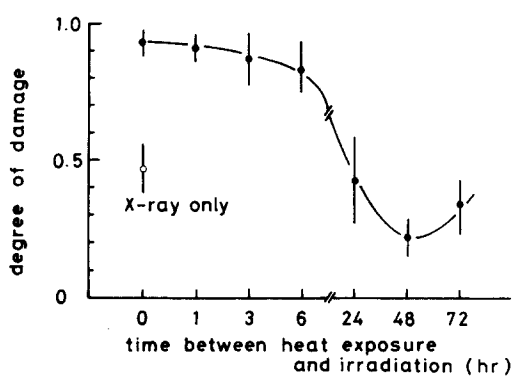

Fig. 6. Synergistic effect of X-irradiation and hyperthermic treatment. Open circle indicates the degree of damage by X-rays alone.

interval was more than 8–12 hr. At an interval of 48 hr, the decreased effect was smaller than that induced by X-irradiation alone and thereafter returned to the X-irradiation level.

DISCUSSION

Previous studies on hyperthermic damage to normal tissues have been performed by exposing animal feet to heat and the damage to the skin examined [5, 6]. This system is too complicated for analysis of damage to normal tissues, because damage to the skin of the feet may result from damage of inner tissues as well. The system described in the present work is, in the author's opinion, preferable and more convenient, as it allows the treatment of the skin alone. As the temperature of treated skin was constant at about 1°C below that of water for live as well as dead animals, the diffusion of heat through blood flow must be negligibly small.

As the isoeffect curves were expressed according to equation (1), the hyperthermic dose for isoeffect is in proportion to t/b^T. The resulting value for b of 0.51 indicates that an increase in temperature of 1°C is equivalent to decreasing the exposure time by half and was consistent with values estimated from data of cultured cells and experimental tumors: 0.49 (Chinese hamster cells [11]), 0.53 (pig kidney cells [12]), 0.5 (sarcoma-180 in SWR mice [13]). This value corresponds with results of others [11] who suggested that it may be correlated with the activation energy of protein inactivation [11]; therefore, thermal damage to the skin might be due to inactivation of protein in cells constituting the skin.

The scoring systems for X-ray damage to the skin reported previously [14, 15] were designed to include a sufficient reliable relationship between the dose of X-rays and the degree of damage. The assignment of grades of damage was possible on the basis of long-standing experience in this field. The initial observation in the present experiments indicated that the peak reaction of the skin damage appeared at the 3rd week after the treatment and was of an entirely different aspect than X-ray damage. Therefore, the degree of the damage was quantified according to the frequencies of occurrence of damage for which the RIDIT system was chosen [8]. This system is preferable in situation

where one is faced with an entirely new set of symptoms [16]. In addition, precautions were taken to insure that the stimuli (i.e. the various combinations of exposure times and temperatures which make a "heat dose") were homogeneously distributed.

The mechanism of cell killing by hyperthermia differs from that of ionizing irradiation. For instance, cells which are in S phase or which are hypoxic are generally considered as being radiation-resistant, but sensitive to heat [11, 17–22]. Using both modalities at the same time is therefore of interest. A syngergistic effect on cell killing may indeed be observed when irradiation and hyperthermia are combined. However, the "therapeutic ratio" is not always increased. Robinson *et al.* [5], for instance, reported a therapeutic ratio of 2.1, but Thrall *et al.* [6] found a value of 0.79 under ambient conditions. The present finding that delay of the irradiation for one or two days (Fig. 6) has a slight protective effect on the skin indicates that it might be worthwhile to separate heat treatment and irradiation for some period of time. However, no information is yet available as to what extent this would influence tumor response.

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Differences between Vinblastine and Vincristine in Distribution in the Blood of Rats and Binding by Platelets and Malignant Cells*

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Abstract—*Tritium labeled vinblastine (VLB) and vincristine (VCR) were injected i.p. at a subacutely-toxic dose* (0.25 mg/kg) *into rats (Nb strain). In the first few hours the levels of VLB in the blood were substantially higher than those of VCR; subsequently, however, the levels of blood radioactivity in the case of VLB fell much more rapidly than those of VCR, reaching values well below the latter in* 8–10 hr. *There was a correlation between the different blood labeling profiles of the alkaloids and their binding by platelets—the carriers of a major proportion of the circulating alkaloids.*

In vitro, *platelets, cells isolated from alkaloid sensitive rat lymphomas and cultured L5178Y cells, all took up VLB much more rapidly than VCR; when restored to alkaloid free medium the cells readily released VLB but, in contrast, retained VCR tenaciously. In binding to cells, VLB and VCR have similar equilibrium association constants and are apparently bound to common receptors.*

It is suggested the preferential retention of VCR by cells may be an important factor underlying the greater toxicity and oncolytic potency of VCR.

INTRODUCTION

VINBLASTINE (VLB) and vincristine (VCR), cytotoxic alkaloids isolated from the plant *Catharanthus roseus* [1, 2], are used extensively in the treatment of certain types of cancer [3, 4]. The two compounds are very similar in structure (Fig. 1) and apparently also in their primary biological action. Thus the toxicity of VLB and VCR can in many cases be linked to disturbances in intracellular microtubular structures resulting from the binding of the alkaloids to a common target molecule, tubulin, the protein subunits from which microtubules are assembled [5–9]. In view of their structural and biological similarities it is of considerable interest that in their use as anticancer agents VLB and VCR differ markedly in their spectra of oncolytic activity, the dosages which are tolerated and the symptoms of toxicity they elicit. VLB, for example, is one of the more useful drugs for treating Hodgkin's disease but has only minimal effects against acute leukemia; VCR, on the other hand, is a valuable drug in the treatment of acute leukemia, particularly in children, and is also effective against certain lymphomas and neuroblastomas [3, 4]. VCR is substantially more toxic to the host than VLB (e.g. tolerated dose for adults: VCR, 25 μg/kg; VLB, 150 μg/kg). The main dose limiting factor in the use

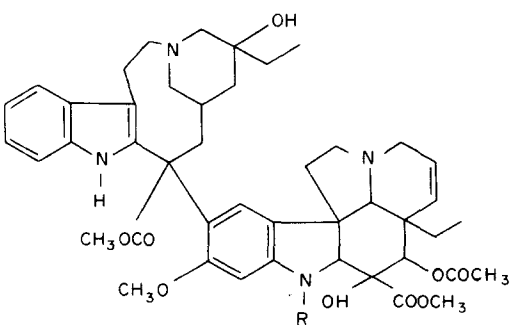

Fig. 1. Vinblastine, R=—CH_3; *vincristine, R*=—CHO.

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†Research Associate, Medical Research Council of Canada.

of VCR is neurotoxicity which may be manifested as a severe neuropathy; the dose of VLB on the other hand is usually limited by its toxic effects on the hematopoietic system (e.g. bone marrow depression, leukopenia) [3, 4].

The biochemical and biological evidence presently available does not provide an explanation of the marked differences in the toxic and oncolytic properties of VLB and VCR. The differences may presumably arise from a number of variables, including the delivery of the administered alkaloids to the tissues and cells with which they interact, the alkaloid concentrations to which the tissues and cells are exposed, the transport of the alkaloids across the plasma membrane to intracellular receptors (e.g. tubulin) and the affinity of the receptors for the alkaloids. The identification and quantitation of the pharmacological, cytological and biochemical parameters which mediate the effects of the alkaloids may lead to a better understanding of their mechanisms of action and to an explanation of the differences between them. In the present study we have compared the pharmacokinetics of distribution of VLB and VCR in the peripheral blood of rats and the binding and release of the alkaloids by alkaloid sensitive cells. The cell types used have included blood platelets (which previous studies indicate may play a role in the transport of the alkaloids in the body [10, 11]) and cells isolated from a line of rat lymphomas which in their response to treatment with the alkaloids resemble certain human malignancies [12, 13]. Marked differences have been found between the two alkaloids both in the pharmacokinetics of their distribution in the blood and in their binding and release by cells. It is suggested that the differences in the cell binding properties of the alkaloids may account, at least in part, for some of the differences in their cytotoxic and oncolytic properties.

MATERIALS AND METHODS

Alkaloids

VLB sulfate and VCR sulfate were gifts from Eli Lilly & Co., Indianapolis, Ind., U.S.A.

Preparation of tritiated VLB and VCR

Tritiated VLB ([^{3}H]VLB) was prepared from VLB by proton exchange with tritiotrifluoroacetic acid and purified by procedures already described [11]. Tritiated VCR ([^{3}H]VCR) was prepared from VCR by the same method and purified by successive chromatography on columns of alumina and cellulose phosphate [10, 14]. The radiochemical purity of the tritiated alkaloids, based on co-chromatography with carrier compounds and isotopic dilution analysis, was: [^{3}H]VLB, 97.8%; [^{3}H]VCR, 95.0%. Their specific radioactivities were: [^{3}H]VLB, 1.4×10^9 dis/min/mg; [^{3}H]VCR, 1.6×10^9 dis/min/mg. The biological activity of the tritiated alkaloids, e.g. inhibition of cell replication, metaphase arrest in cultures of L5178Y cells, was the same as that of the corresponding non-radioactive compounds.

Rats

The animals used in this study were Nb strain rats bred in the animal colony of the Cancer Research Centre, University of British Columbia [15]. Male rats, wt 225 ± 25 g, were given i.p. injections of the tritiated alkaloids (0.25 mg/kg) dissolved in 1 ml of 0.9% NaCl solution.

Tumors

Lymphomas of the Node lymphoma No. 2 line were maintained by subcutaneous transplantation in the neck region of Nb strain rats. As reported elsewhere [13, 15] this lymphoma grows rapidly to give well defined masses with minimal necrosis and hemorrhage, and even when far advanced responds well to treatment with either VLB or VCR, regressing rapidly until no longer palpable. On a mg/kg basis VCR is 2–3 times more effective than VLB [13].

Blood sampling and fractionation

Blood was sampled from the tail or heart of the rats [10]. Blood plasma, WBC and RBC fractions were obtained by centrifuging citrated blood for 10 min at 4300 ***g*** in polyethylene tubing and separating the fractions by cutting the tubing with a razor blade as previously described [10, 11].

Preparation of platelet rich plasma

Platelet rich plasma (PRP) was prepared from heart blood of lightly etherized rats by the method of Aster and Jandl [16]. The blood (8 ml) was drawn into a 10 ml plastic syringe containing 1.6 ml of 0.15 M citrate (pH 4.6) as anticoagulant. After mixing, the citrated blood was expelled into a plastic centrifuge tube, allowed to stand

at room temperature for 20 min and then centrifuged for 15 min at 275 ***g***. The supernatant and WBC layer were removed with a siliconized pipette as one fraction which was then centrifuged for 5 min at 400 ***g*** to pack down erythrocytes and leukocytes. The supernatant was removed and used as "platelet rich plasma". The platelet count was $1.0 \pm 0.2 \times 10^6/mm^3$; red cell and leukocyte contamination was very low.

Preparation of cell suspensions from rat lymphomas

Rats bearing rapidly growing transplanted Node lymphomas (1.5–2.5 cm dia) were etherized and the tumors excised. Portions of the tumors, free of necrotic and hemorrhagic areas, were minced with scissors in a mixture of Hanks' balanced salt solution (6 vol) and calf serum (4 vol), buffered at pH 6.8 with citrate (0.015 M). The resulting suspension was filtered through stainless steel gauze to remove pieces of tumor tissue giving a suspension of cells which was usually contaminated with a substantial number of platelets. In view of the high affinity of platelets for VLB and VCR and the errors which might then arise in studying alkaloid-tumor cell binding, it was necessary to reduce platelet contamination to a minimum. This was done by repeated centrifugation (4–5 times) of the cells for 5 min at 300 ***g*** followed by resuspension of the cell pellet in fresh Hanks' salt solution–calf serum mixture. After the final centrifugation the cells were resuspended in Fischer's medium (Hyland, L.A., Calif., U.S.A.) containing calf serum (10%). This procedure gave a suspension of single refractile cells of which over 90% appeared to be viable on the basis of eosin exclusion; when the cell suspension was incubated with VLB at an antimitotic concentration (0.05 μg/ml) a high proportion of the cells accumulated at metaphase, indicating the presence of actively cycling cells.

Binding and release of tritiated alkaloids by platelets and tumor cells in vitro

Platelet rich plasma (*ca.* 10^9 platelets/ml), suspensions of cells isolated from rat lymphomas (*ca.* 15×10^6 cells/ml) and cultures of L5178Y mouse lymphoma cells [17] (*ca.* 5×10^6 cells/ml) were incubated at 37°C with [^{3}H]VLB and [^{3}H]VCR in stoppered siliconized glass vials rotating at 4 rev/min. At intervals throughout the incubation portions (*ca.* 100mg) of the suspensions were withdrawn into 7 cm lengths of polyethylene tubing (i.d. 0.066 in.). The tubes, sealed at one end, were centrifuged (7 min at 4300 ***g***) and the cell pellet separated from the supernatant by cutting the tubing immediately above the pellet [10, 11]. Both pellet and supernatant were radioassayed as described below. The pellet radioactivity was corrected for the small amount of supernatant radioactivity present in the pellet fraction; this correction was less than 5% of the radioactivity present in the supernatant.

When it was necessary to restore platelets to alkaloid free plasma, the suspensions were centrifuged for 20 min at 1700 ***g***, the supernatants carefully removed and the packed cells then gently resuspended in the original volume of alkaloid free, citrated plasma (pH 6.5). The radioactivity in the very small amount of supernatant retained in the pellet accounted for less than 2% of the total radioactivity in the pellet. Platelet losses during the procedure were low (less than 5%). When restoring tumor cells to alkaloid free medium, the cell suspensions were diluted with an equal volume of alkaloid free medium, centrifuged for 5 min at 300 ***g*** and the packed cells resuspended in the original volume of alkaloid free medium. Residual supernatant radioactivity accounted for less than 1% of the pellet radioactvity. Cell losses were small (less than 5%).

Radioassay methods

For the radioassay of blood, blood fractions, platelets, cells and supernatants, samples (up to 100 mg) were incubated in scintillation vials with 0.5 ml of 1N NaOH at 37°C for 24 hr and then if necessary decolorized with hydrogen peroxide [10]. Ten millilitres of PCS solubilizer (Amersham/Searle, Illinois, U.S.A.) containing 4% glacial acetic acid were then added to the vials and the radioactivity determined by liquid scintillation counting. Duplicate samples were counted to an accuracy of ±3% at the 95% confidence level and corrected for quenching.

The percentage of the radioactivity in a sample which was due to [^{3}H]VLB or [^{3}H]VCR was determined by isotopic dilution analysis as follows. A known amount of the sample was homogenized at pH 2–3 with a large known excess of purified carrier VLB or VCR. After removing small aliquots for assaying the total radioactivity in the sample, the alkaloid in the rest of the homogenate was extracted into benzene at pH 7 and then purified chromatographically using at least 3 systems. The latter

included elution from columns of alumina, cellulose phosphate or carboxymethyl cellulose using procedures already described [10. 11. 14] and also thin layer chromatography on alumina (Eastman Kodak No. 6063) run with acetone or ethylacetate/*iso*amyl alcohol (10:1) and on silica gel (Eastman Kodak No. 6060) run with acetone or ethylacetate/ethanol (1:1). In running the columns, multiple fractions were collected during the period when the carrier was leaving the column in order to verify that the radioactivity elution profile accurately matched that of the optical density [14]. The specific radioactivity of the recovered carrier alkaloid was determined from its radioactivity and u.v. absorption, and used to calculate the percentage of radioactivity in the original sample which was due to [^{3}H]VLB or [^{3}H]VCR.

RESULTS

Radioactivity in blood and blood fractions of rats given [^{3}H]*VLB and* [^{3}H]*VCR*

The levels and distribution of radioactivity in the peripheral blood of rats given single i.p. injections of [^{3}H]VLB and [^{3}H]VCR at a nonacutely-toxic dose (0.25 mg/kg) are shown in Fig. 2. The whole blood radioactivity profiles of the 2 alkaloids (Fig. 2A) have certain features in common. For example, in both cases the radioactivity appeared in the blood almost immediately after the injection, rose sharply to a peak value and then declined rapidly to much lower values. However, marked differences were also found: while the levels of radioactivity in the blood of rats given [^{3}H]VLB were initially substantially higher than those in the blood of rats given [^{3}H]VCR, in the case of VLB they fell much more extensively and after 8 hr were well below those in the blood of rats given [^{3}H]VCR.

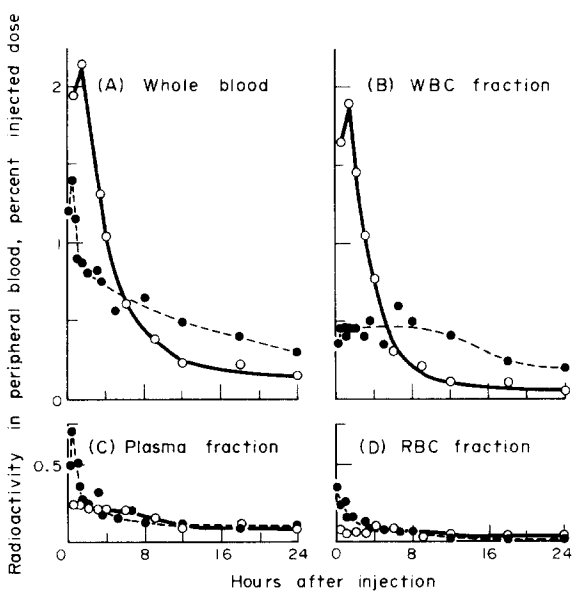

Fig. 2. Distribution of radioactivity in peripheral blood of rats given i.p. injections of [^{3}H]*VLB* (0.25 mg/kg; 3.5 × 10^8 dis/min/kg) *and* [^{3}H]*VCR* (0.25 mg/kg; 4.0 × 10^8 dis/min/kg). *Plasma, WBC and RBC fraction were obtained by centrifuging citrated tail blood samples at* 4300 *g* ("*Methods*"). *Radioactivity in whole blood* (*A*), *WBC fraction* (*B*), *plasma fraction* (*C*) *and RBC fraction* (*D*) *is expressed as percent of injected radioactivity, taking blood weight as* 7.5% *of body weight.* [^{3}H]*VLB*, ○——○, [^{3}H]*VCR*, ●--●. *Points are averages of results from at least* 3 *rats.*

A comparison of the labeling of the WBC fraction (Fig. 2B) shows that in the first few hours after the injection of [^{3}H]*VLB* this fraction was much more highly labeled than after the injection of [^{3}H]VCR. However, as time progressed this situation was reversed; in rats injected with [^{3}H]VLB the WBC radioactivity decreased sharply from its initial peak value, whereas in rats given [^{3}H]VCR the WBC radioactivity remained essentially constant for about 12 hr and thereafter declined only slowly. The labeling of the plasma fraction (Fig. 2C) was very similar for the 2 alkaloids except for a period of about 1 hr immediately after the injection. In this period the plasma radioactivity levels in the rats given [^{3}H]VCR were substantially higher than in those given [^{3}H]VLB. The levels of radioactivity in the RBC fraction (Fig. 2D) were very low except for a short period immmediately following the injection of [^{3}H]VCR; the temporary higher levels of [^{3}H]VCR radioactivity in this fraction are probably due to small amounts of entrapped plasma.

It can be seen from Fig. 2 that the WBC and plasma fractions together account for most of the radioactivity in the blood after the administration of either VLB or VCR. In the first 8–10 hr after injecting [^{3}H]VLB most of the circulating radioactivity was present in the WBC fraction. With VCR on the other hand, a substantial proportion of the circulating radioactivity was also present in the plasma, especially in the first few hours, but as time progressed most of the radioactivity was confined to the WBC fraction. It appears from the results that the differences betwen VLB and VCR in the labeling of the peripheral blood can to a major extent be accounted for by the labeling patterns of the WBC fraction.

It was shown by isotopic dilution analysis

("Methods") that, even 24 hr after the injection of [^{3}H]VCR, over 90% of the radioactivity in the WBC fraction was due to the unchanged alkaloid and furthermore using blood cell preparation methods [10], that as least 60% of the alkaloid in this fraction was associated with platelets. It appears therefore that platelets are the main cellular carriers of VCR in the peripheral blood of rats. We have already shown in a previous study [10] that in the peripheral blood, platelets are also the main cellular carriers of VLB.

Binding and release of [^{3}H]*VLB and* [^{3}H]*VCR by blood platelets* in vitro

Platelet rich plasma was incubated at 37°C with [^{3}H]VLB and [^{3}H]VCR at various concentrations. The binding of the alkaloids by platelets at 2 alkaloid concentrations is shown in Fig. 3. Very similar results were obtained at alkaloid concentrations up to 2 μg/ml. The lower concentration in Fig. 3 (0.05 μg/ml) is of the same order as that found in the plasma of rats given subacutely-toxic doses of VLB [13]. As found in a previous study [11], [^{3}H]VLB was rapidly taken up by platelets and in about 1 hr the amount of platelet bound alkaloid had reached a plateau. [^{3}H]VCR was bound by platelets at a much lower rate than VLB but eventually (after 8–10 hr) reached the same plateau as VLB at a given concentration. When the plateaus were reached, a substantial portion of the total alkaloid present had been taken up by the platelets, e.g. *ca.* 70 and 50% at alkaloid concentrations of 0.05 and 0.44 μg/ml, respectively. This demonstrates the marked ability of the platelets to concentrate the alkaloids from the plasma.

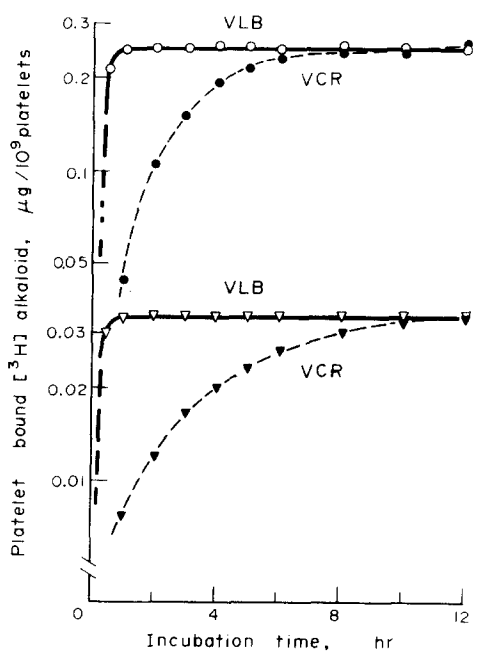

Fig. 3. Binding of [^{3}H]*VLB and* [^{3}H]*VCR by platelets* in vitro. *Aliquots of PRP containing* ca. 10^9 *platelets*/ml *were incubated at* 37°C *with* [^{3}H]*VLB* (0.44 μg/ml. ○——○; 0.05 μg/ml, ▽——▽) *and* [^{3}H]*VCR* (0.44 μg/ml. ●--●; 0.05 μg/ml, ▼--▼). *The amount of platelet bound alkaloid was determined at the times shown on centrifuged samples* ("*Methods*").

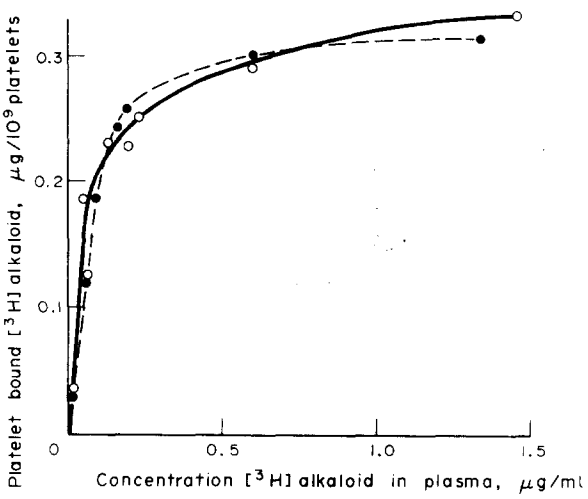

Fig. 4. Plots of amounts of [^{3}H]*VLB and* [^{3}H]*VCR bound at equilibrium by platelets vs concentrations of unbound alkaloid in the plasma. Aliquots of PRP* (*containing* 10^9 *platelets*/ml) *were incubated with the tritiated alkaloids at various concentrations. The amounts of platelet bound alkaloid and the alkaloid concentrations in the plasma were determined at intervals* ("*Methods*") *until equilibrium was established* (*usually within* 2 hr *for VLB and* 8–10 hr *for VCR; see Fig.* 3). *VLB*, ○——○; *VCR*, ●--●.

In Fig. 4 the amounts of alkaloid bound by platelets when a plateau had been reached (*ca.* 2 hr for VLB, 8–10 hr for VCR) are plotted against the concentrations of unbound alkaloid in the plasma. It can be seen that at the higher alkaloid concentrations the platelets became saturated with the alkaloids and also that over the entire concentration range the curves for VLB and VCR are essentially identical. Since the platelets bound equal amounts of VLB and VCR at saturation, it appears that the same number of binding sites are available for each alkaloid. Furthermore the close similarity in the curves indicates that the equilibrium association constants of the 2 alkaloids for the platelets are very similar.

When platelets which had taken up VLB and VCR were restored to alkaloid free plasma, there was a considerable difference in the ease with which the 2 compounds were released (Fig. 5). Platelets labeled with

[^{3}H]VLB readily released the alkaloid until equilibrium was once more established between the VLB bound by the platelets and that in the plasma (Fig. 5, upper panel). When resuspended for a second time in alkaloid free plasma, the platelets released

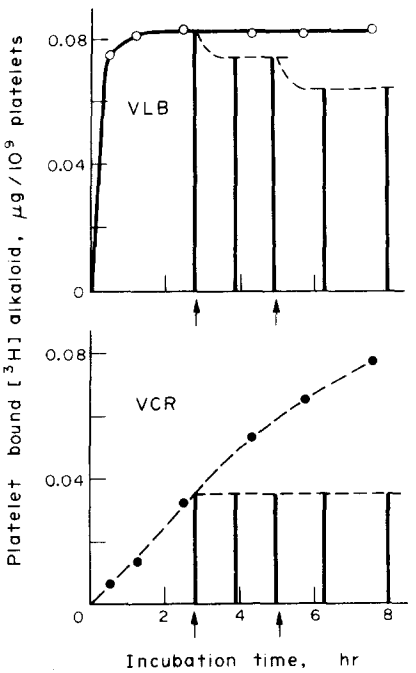

Fig. 5. Retention of [^{3}H]*VLB and* [^{3}H]*VCR by platelets on restoration to alkaloid free plasma. Aliquots of PRP were incubated at* 37°C *with* [^{3}H]*VLB* (0.11 μg/ml) *and* [^{3}H]*VCR* (0.11 μg/ml). *At intervals, samples were taken from the two suspensions for determination of the amounts of platelet associated alkaloid ("Methods"): VLB,* ○——○; *VCR,* ●--●. *After* ca. 3 hr (*1st arrow*) *platelets from a portion of each suspension were restored to alkaloid free plasma ("Methods") and the radioactivity retained by these platelets on further incubation was determined at* 2 *intervals (vertical bars,* hr 4 *and* 5). *At the time indicated by the 2nd arrow the platelets were once more resuspended in alkaloid free plasma and, after further incubation, again assayed for platelet associated alkaloid (vertical bars,* hr 6 *and* 8). *Platelet losses as a result of the manipulations were small (less than* 5%); *the results have been normalized for* 10^9 *platelets.*

a further amount of VLB, again establishing an equilibrium between bound and free alkaloid. The results indicate that the binding of [^{3}H]VLB by platelets is readily reversible, confirming conclusions reached in a previous study [11]. In contrast, platelets which had been labeled with [^{3}H]VCR released very little of the bound alkaloid when restored to alkaloid free plasma; even after a second resuspension in alkaloid free plasma and prolonged incubation the platelets retained essentially all the VCR initially bound (Fig. 5, lower panel).

Labeling of the WBC fraction in rats given i.v. injections of platelets prelabeled with [^{3}H]*VLB and* [^{3}H]*VCR*

The finding that platelets are the main cellular carriers of VLB and VCR in the peripheral blood of rats suggested that the differences in the labeling patterns of the WBC fractions in rats given the tritiated alkaloids (Fig. 2B) might be related to the differences in the alkaloid binding properties of platelets as found in the *in vitro* studies (Figs. 3 and 5). One aspect of this suggestion was investigated by giving rats intravenous injections of platelets which had been prelabeled *in vitro* with [^{3}H]VLB or [^{3}H]VCR and then determining the levels of radioactivity in the blood and its distribution among the blood fractions for 1–2 days. During this period essentially all the blood radioactivity was found to be concentrated in the platelet containing WBC fraction; the amounts of radioactivity in the plasma and RBC fraction were negligible. Figure 6 shows the percentage of injected radioactivity present in the WBC fraction of the rats, calculated on the basis that blood volume is 7.5% of body weight. In the first few min after the injection of platelets labeled with [^{3}H]VLB or [^{3}H]VCR, most of the injected radioactivity was in each case found to be present in the WBC fraction. Since the platelets were injected into the jugular vein and the blood was

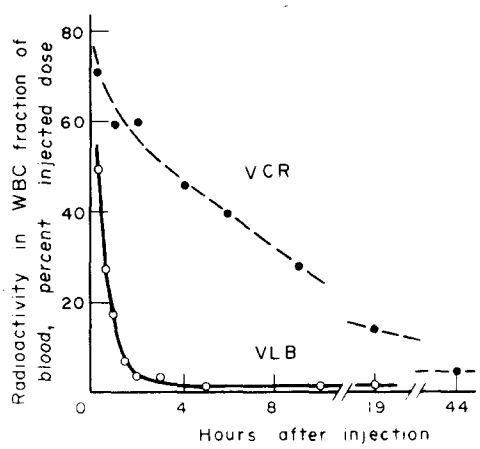

Fig. 6. Radioactivity of WBC fraction of peripheral blood of rats given i.v. injections of platelets prelabeled with [^{3}H]*VLB and* [^{3}H]*VCR. Platelets were prelabeled by incubating PRP at* 37°C *for* 3 hr *with* [^{3}H]*VLB* (0.3 μg/ml) *and* [^{3}H]*VCR* (0.9 μg/ml), *collected by centrifugation and resuspended in small volumes of alkaloid free plasma. Platelets* (ca. 3×10^9) *labeled with* [^{3}H]*VLB* (0.47 μg) *and* [^{3}H]*VCR* (0.48 μg) *were injected into the jugular vein of rats. The radioactivity in the WBC fraction of the total blood of the rats (calculated on the basis that blood weight is* 7.5% *of the body weight) is given as a percentage of injected radioactivity (means of* 2 *determinations). VLB,* ○——○; *VCR,* ●--●.

sampled from the tail, it appears that the platelets had been rapidly dispersed in the circulation. The rates at which the levels of WBC radioactivity (and hence also the levels of radioactivity in the blood) declined from their initial high values were markedly different for the 2 alkaloids. Thus after the injection of [^{3}H]VLB labeled platelets the WBC radioactivity declined very rapidly and within 2 hr only a few per cent of the injected radioactivity remained in the blood; in contrast, the levels of radioactivity in the WBC fraction after the injection of [^{3}H]VCR labeled platelets declined much more slowly and even after 10 hr over 25% of the injected radioactivity was still present in the circulation. It may be noted that the decline in the radioactivity of the WBC fractions will reflect not only the rates at which the [^{3}H]alkaloids are released from the platelets but also the loss of alkaloid labeled platelets from the circulation, e.g. as a result both of normal platelet turnover and the exchange of platelets in the circulation with those elsewhere in the body. On the assumption that the rate of platelet loss from the peripheral blood is the same for platelets labeled with [^{3}H]VLB as for platelets labeled with [^{3}H]VCR, it seems very probable that the difference between the 2 alkaloids in the decline of the WBC radioactivity (Fig. 6) is due largely to a ready release of VLB and a contrasting tenacious retention of VCR by platelets *in vivo*.

Binding and release of [^{3}H]*VLB and* [^{3}H]*VCR by tumor cells* in vitro

The binding and release of [^{3}H]VLB and [^{3}H]VCR by tumor cells *in vitro* was studied using suspensions of cells isolated from rat Node lymphomas and suspensions of cultured L5178Y mouse lymphoma cells. Figure 7 shows the labeling of the cells isolated from rat lymphomas when incubated with [^{3}H]VLB and [^{3}H]VCR at two concentrations, the lower of which (0.05 μg/ml) is in the range of that found in the plasma of rats given a therapeutically effective dose of VLB [13]. Both alkaloids were taken up by the cells from the medium at rates which for each alkaloid were proportional to its concentration. At a given alkaloid concentration, VLB was bound by the cells much more quickly than VCR. Thus, whereas the amount of [^{3}H]VLB bound by the cells reached a plateau in *ca.* 50 min, at least 5 hr elapsed before the amount of VCR bound approached a plateau. The

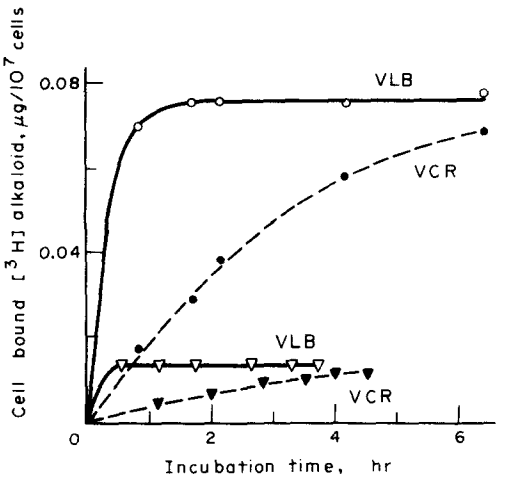

Fig. 7. Binding in vitro *of* [^{3}H]*VLB and* [^{3}H]*VCR by cells isolated from rat lymphomas. Aliquots of cell suspensions prepared from rat lymphomas* (ca. 15×10^6 *cells*/ml: "*Methods*") *were incubated at* 37°C *with* [^{3}H]*VLB* (0.46 μg/ml, ○——○; 0.05 μg/ml, ▽——▽ *and* [^{3}H]*VCR* (0.46 μg/ml, ●--●; 0.05 μg/ml, ▼--▼). *The amount of cell bound alkaloid was determined at the times shown on centrifugated samples* ("*Methods*").

amount of VCR eventually bound, however, quite closely approximated the amount of VLB bound at the same concentration. Isotopic dilution analysis showed that the cell associated radioactivity was due almost entirely to the unchanged alkaloids, e.g. after 5 hr incubation with the alkaloids, 85 and 95% of the cell bound radioactivity was due to [^{3}H]VLB and [^{3}H]VCR, respectively.

When isolated lymphoma cells, which had been prelabeled by incubation with [^{3}H]VLB and [^{3}H]VCR, were restored to alkaloid free medium and further incubated, they released the two alkaloids at markedly different rates. The results of a typical experiment are shown in Fig. 8. The cells which had been prelabeled with [^{3}H]VLB released a substantial amount of the cell bound alkaloid each time they were reincubated in fresh alkaloid free medium and after 6 such treatments the cells retained only 5% of the alkaloid initially taken up. In contrast, the cells prelabeled with [^{3}H]VCR released very little radioactivity after each reincubation in alkaloid free medium and even after 6 medium changes the cells retained over 75% of the [^{3}H]VCR originally bound. Figure 8 also shows that even if cells have initially taken up much more VLB than VCR, on lowering the extracellular alkaloid concentration VLB is released so readily that eventually the amount of VCR retained by the cells can greatly

exceed that of VLB. For example, although on incubation for 100 min with the [^{3}H]alkaloids, the tumor cells took up only one-third as much VCR as VLB, after 6 reincubations in alkaloid free medium they retained 6 times as much VCR as VLB.

Cultured L5178Y mouse lymphoma cells behaved very similarly to the cells isolated from the rat lymphomas with respect to the uptake and release of the tritiated alkaloids, i.e. [^{3}H]VLB was taken up quickly and readily released; [^{3}H]VCR was taken up slowly and released very slowly (data not included).

DISCUSSION

The present study has shown that VLB and VCR differ markedly both in the pharmacokinetics of their distribution in the peripheral blood of rats and in their binding by cells. The differences between the alkaloids in their uptake and release by cells seem to be particularly important since they appear to provide explanations not only of the differences in the pharmacokinetic distribution of the alkaloids, but also of some of the differences in the toxic and oncolytic properties of the alkaloids.

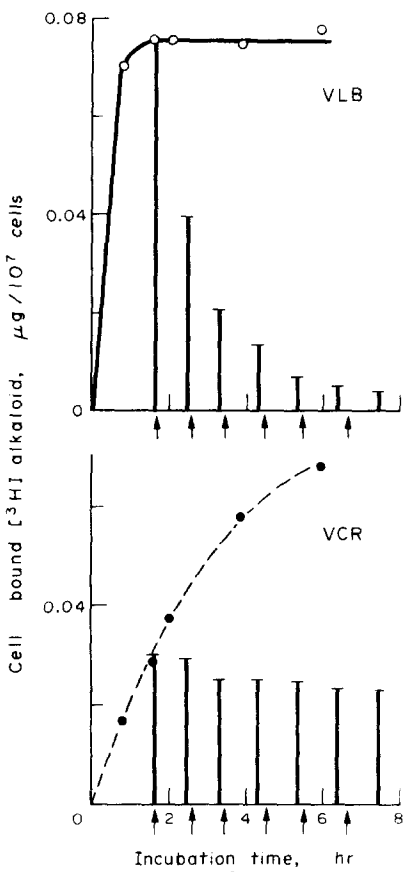

Fig. 8. Retention of [^{3}H]VLB and [^{3}H]VCR by isolated rat lymphoma cells on restoration to alkaloid free medium. Aliquots of cell suspensions prepared from rat lymphomas ("Methods") were incubated at 37°C with [^{3}H]VLB (0.46 μg/ml) and [^{3}H]VCR (0.46 μg/ml). The amounts of cell bound alkaloid were determined at intervals up to 6 hr (VLB, O——O; VCR, ●--●). After labeling the cells for 100 min a portion of each suspension was taken for determining the amounts of alkaloid retained by the cells when restored to alkaloid free medium ("Methods"). At each of the times indicated by the arrows the cells were resuspended in alkaloid free medium and incubation continued; the vertical bars indicate the amounts of alkaloid retained by the cells 50 min after each resuspension. The total loss in cell numbers resulting from the multiple resuspensions was less than 10%; the results have been normalized for 10^7 cells.

The marked differences observed in the pharmacokinetic distribution of VLB and VCR in the peripheral blood of rats (Fig. 2) can be correlated with differences in the alkaloid binding properties of blood platelets which, as shown in this and a previous study [10], are the main cellular carriers of VLB and VCR in the peripheral blood. It seems very likely, for example, that the high concentrations of VLB which are found almost immediately in the WBC fraction (Fig. 2B) and the comparatively low labeling of the plasma by [^{3}H]VLB (Fig. 2C) reflect the high rate at which platelets are able to concentrate VLB from the plasma, as found *in vitro* (Fig. 3). Furthermore, the higher levels of VCR initially found in the plasma and the relatively lower amounts of VCR in the WBC fraction are consistent with the lower rate at which platelets take up VCR as compared to VLB (Fig. 3). The rapid decline in the levels of VLB in the WBC fraction and the contrasting sustained levels of VCR in this fraction (Fig. 2B) can likewise be explained by the ease with which platelets release VLB and the tenacity with which they retain VCR, as found on restoration of labeled platelets to alkaloid free plasma *in vitro* (Fig. 5) and also *in vivo*, when platelets labeled with VLB and VCR were injected intravenously into rats (Fig. 6).

The marked differences between VLB and VCR in their rates of uptake and release by cells were found not only with blood platelets but also with cells isolated from rat lymphomas and cultured L5178Y mouse lymphoma cells. Thus all the cells took up VLB much more quickly than VCR (Figs. 3 and 7) and on restoration to alkaloid free medium readily released VLB but tenaciously retained VCR (Figs. 5 and 8). In view of the similarity in the alkaloid binding properties of cells of such diverse types as platelets (with no nucleus and comparatively simple metabolism) and replicating tumor cells, it seems likely that the mechanisms involved in the binding of the

alkaloids by cells—including their ability to differentiate between the 2 alkaloids—are common to a variety of cell types.

The use of platelets as comparatively simple models for studying the interaction of Vinca alkaloids with their cellular receptors has been indicated by a previous study [11] in which it was shown that in binding [^{3}H]VLB, platelets behave as if they have saturable receptors with a high affinity for the alkaloid; the displacement of platelet bound [^{3}H]VLB by non-radioactive VCR could tentatively be explained by a kinetic model in which VCR competed for the same binding sites as VLB but at a much lower rate. The present study, in which [^{3}H]VCR as well as [^{3}H]VLB has been used, provides strong support for this model. Thus it shows that even though, at a given alkaloid concentration, platelets may eventually take up as much VCR as VLB, they bind VCR at a much lower rate than VLB (Fig. 3). The finding that at saturation platelets bind equal amounts of each alkaloid, together with our previous observation that [^{3}H]VLB is displaced by VCR [11], strongly suggests that in platelets the 2 alkaloids do in fact have the same binding sites. In this connection it may be noted that Bleyer *et al.* [18] have concluded on the basis of competition studies that VLB and VCR have common binding sites in L1210 leukemia cells. It also appears that while VLB and VCR differ markedly in the rates at which they are taken up by platelets, they nevertheless have similar equilibrium association constants for platelets as indicated by the results in Figs. 3 and 4. VLB and VCR appear also to have very similar equilibrium association constants for cells isolated from rat lymphomas and for L5178Y cells, since, at a given alkaloid concentration, the cells eventually take up about the same amounts of each alkaloid (Fig. 7). It is of considerable interest that VLB and VCR should differ so markedly in their rates of uptake and release by platelets and tumor cells even though they have similar equilibrium association constants for the cells and apparently common receptors.

There is substantial evidence from other work that a major receptor for the biologically active Vinca alkaloids, including VLB and VCR, is "tubulin"—the protein subunits of cellular microtubules [5–7]. Although in the present study the specific nature of the cellular alkaloid receptors has not been investigated, it may be noted that exposure of the cells to the alkaloids resulted in pronounced disturbances in microtubular structures—indicative of tubulin–alkaloid interaction—i.e. disruption of the mitotic spindle in the tumor cells and of the circumferential bundle of microtubules in the platelets [10, 19]. The binding of VLB and VCR to isolated tubulin in certain respects resembles their binding to intact cells, e.g. both alkaloids have a high affinity for tubulin with association constants that are of the same order [20, 21] and they also appear to compete for the same binding sites on the tubulin [22, 23]. In spite of these similarities, however, the intact cells and tubulin differ markedly in the rates at which they bind VLB and VCR. Thus whereas VCR is bound much more slowly than VLB by platelets and tumor cells (Figs. 3 and 7), both alkaloids have been reported to bind rapidly to isolated tubulin, reaching equilibrium after only a few minutes incubation, at which point equal amounts of the compounds were bound [20]. This suggests that if the alkaloid binding properties of tubulin in intact cells are the same as *in vitro*, then the different rates at which VLB and VCR are taken up by cells are not determined primarily by differences in their binding to tubulin but are due instead to a rate controlling process which differentiates between the 2 alkaloids and which is not directly related to the interaction of the alkaloids with tubulin. It is tempting to speculate that the difference between VLB and VCR in their uptake (and possibly their release) by cells may stem from differences in the rates at which they are transported across the plasma membrane.

The pharmacokinetic distribution of VLB and VCR in the blood and the binding of the alkaloids by cells are, almost certainly, important variables mediating the toxic and oncolytic effects of these compounds. This raises the question of the extent to which *differences* in the latter are related to differences in the levels and distribution of VLB and VCR in the peripheral blood (Fig. 2). Although the levels of VCR in the plasma were for a short period substantially higher than those of VLB (Fig. 2C), it appears unlikely that during this period cells will accumulate even as much VCR as VLB in view of the lower rate at which they take up VCR. It is doubtful therefore if the transient higher levels of

VCR in the plasma account to a major extent for the greater toxicity and oncolytic potency of VCR. Regarding the transport of the alkaloids in the WBC fraction, it is possible that in the case of VLB the platelets may, as suggested elsewhere [11], play a role in the delivery of this alkaloid to tissues, in view of the readiness with which they take up and release VLB. On the other hand it is rather doubtful if platelets play a significant role in the delivery of VCR, in view of the tenacity with which they retain VCR. While the overall effect of the differences in the transport of VLB and VCR in the blood is not entirely clear, they do not appear to provide an adequate explanation of the differences in the toxic and oncolytic properties of the 2 alkaloids.

While, as indicated above, there is substantial evidence that the biological effects of VLB and VCR stem primarily from their binding to tubulin [5–9], the relatively minor differences in the interaction of the alkaloids with tubulin do not appear to account for the marked differences in the toxic and oncolytic properties of the compounds. In fact, Himes *et al.* [24] have suggested that the latter are likely to be determined by processes other than the interaction of VLB and VCR with tubulin *per se*, since the alkaloids not only have very similar tubulin binding properties but are also equally effective in inhibiting the polymerization of tubulin *in vitro*. From results in the present study it seems likely that at least a partial explanation of the greater toxicity and oncolytic potency of VCR may lie in the markedly greater tenacity with which cells retain VCR. Thus even though cells, on exposure to the alkaloids, may accumulate less VCR than VLB (e.g. as a result of the lower rate at which VCR is taken up), they will, in the face of falling extracellular alkaloid levels, retain VCR much longer and eventually in greater amounts than VLB (Fig. 8). It may be anticipated that when sensitive cells are exposed to the alkaloids for short periods, as may occur *in vivo*, VCR, as a result of its retention by cells, will continue to exert its toxic effects long after its extracellular levels have fallen to insignificant values; the effects of VLB on the other hand are likely to be less persistent in view of the readiness with which this alkaloid is released by cells when the extracellular alkaloid levels decline (Fig. 8). The differential in the retention of VLB and VCR by cells may assume special significance when asynchronously replicating cells are exposed to the alkaloids. Replicating cells are particularly sensitive to the alkaloids at mitosis and if briefly exposed to the compounds at less sensitive phases of the cell cycle, may retain much more VCR than VLB by the time they reach mitosis. VCR may also, in view of its more sustained retention by cells, have a more adverse effect than VLB on the recovery of cells from microtubular damage, perhaps accounting for its greater neurotoxicity.

In view of the readiness with which VLB is released by cells it may be expected that its toxic effects will be more dependent than those of VCR on the maintenance of elevated alkaloid levels in the immediate environment of cells. In this connection it is of interest that whereas, *in vitro*, cells isolated from rat lymphomas readily release VLB (Fig. 8), nevertheless in the animal the lymphoma has been found to retain the alkaloid long after the levels of VLB in the circulation had fallen to very low levels; it was suggested that the continuing presence of VLB in the tumor might be an important factor underlying the high sensitivity of the lymphoma to treatment with the alkaloid [13]. The mechanism of retention of VLB by the lymphoma *in vivo* is not yet known but may be related to the lymphoid character of the tumor tissue [13]. The difference in the retention of VLB *in vitro* by isolated cells and *in vivo* by the lymphoma raises the possibility that there may be more than one mechanism for maintaining elevated alkaloid levels in "target" tissues, e.g. retention by cells *per se* as found for VCR, and retention by virtue of some special property of the tissue as suggested for VLB in the lymphoma. The existence of alternative mechanisms could help to explain why VLB has a more limited spectrum of oncolytic activity than VCR, and why, for example, it is useful in the treatment of certain lymphomas (e.g. Hodgkin's disease, Nb strain rat lymphomas) but is much less effective than VCR in treating leukemias. Further studies on the mechanism of action of the Vinca alkaloids both at the cellular level and *in vivo* are clearly required.

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Rapid Remission Induction in Adult Acute Non-Lymphoblastic Leukaemia*

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Abstract—*Nineteen out of* 40 *adults with acute non-lymphoblastic leukaemia attained complete remission. All these individuals had a neutrophil count greater than* 1 $\times 10^9$/1 *and a platelet count above* 100×9/1 *within an average of* 16 *days of completing a single* 5-day *course of cytotoxic chemotherapy comprising the epipodophyllotoxin VP*16–213, *cytosine arabinoside and adriamycin. This induction regimen offers an advantage where patients with acute leukaemia have to be treated in general medical wards without protected environments since the short neutropenic period permits early resolution of infection which remains the commonest cause of death during induction.*

INTRODUCTION

THE TREATMENT of acute non-lymphoblastic leukaemia has improved following the introduction of the drugs cytosine arabinoside and daunorubicin or its analogue adriamycin [1]. Although schedules for the administration of these agents vary they are given to achieve bone marrow aplasia which is generally accepted as a prerequisite for the regeneration of normal haematopoietic tissue and therefore complete remission [2]. During this aplastic period the patient is at risk of dying from haemorrhage and infection [3] and some workers, unable to justify this, have advocated using lower doses of antileukaemic drugs. However, such an approach has had an adverse effect on patient survival [4]. In specialised treatment centres aggressive induction regimens are administered to patients housed in reverse-barrier environments [5] and neutrophils [6] and platelet [7] transfusions are readily available. Such comprehensive supportive therapy is the major factor in determining patient recovery from the aplastic phase [5] but, unfortunately, these facilities are costly and not universally available.

In those hospitals where the physician is treating patients with acute leukaemia without the benefit of such specialised facilities a short pancytopenic period may be of advantage by limiting the interval during which they are at risk of dying from infection and haemorrhage. In this context we report our remission induction results obtained in 40 adults treated in a general hospital environment with a combination of the epipodophyllotoxin VP16–213, cytosine arabinoside and adriamycin.

MATERIALS AND METHODS

Forty consecutive previously untreated adults with acute non-lymphoblastic leukaemia were studied between January 1975 and July 1977 (Table 1). Variants such as smouldering leukaemia and blastic transformation of chronic granulocytic leukaemia were not included and no patient was excluded once treatment had commenced. Patients were treated in general medical wards and all received oral allopurinol, kanamycin and nystatin and were given frequent 0.2% Hibitane mouth washes. Pyrexia was, in the absence of a readily identifiable cause such as a recent transfusion, presumed to be due to infection and was treated with a combination of gentamycin, cephalothin and carbenicillin. Granulocytes obtained by filtration leucapheresis were

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Table 1. *Group characteristics*

Parameter	Group 1 (Regenerated)	Group 2a (Died—hypoplastic)	Group 2b (Failed treatment)	Group 2c (Early deaths)
Number	19	9	5	7
Sex:				
Male	9	3	3	2
Female	10	6	1	6
Age: years				
Mean	28.7‡	44.8	30.3	43.4
Range	13–50	19–65	15–62	17–67
Haemoglobin: g/dl				
Mean	8.5	9.72	6.63	8.09
Range	3–15	5–12.1	4–10.5	4.1–14.3
Total White Cell: $\times 10^9/l$				
Mean	12.2*	34.4	41.5	55.5
Platelets: $\times 10^9/l$				
Mean	100.9†	89.4	55.5	96.3

Statistics
Unpaired Student's *t*-test:
*$0.01 < 2P > 0.02$.
†$0.2 < 2P > 0.3$ (not significant).
‡$0.01 < 2P > 0.02$.

transfused if the temperature failed to respond to the antibiotics within 48 hr provided that the patient had an absolute neutrophil count less than $0.5 \times 10^9/l$. Platelets obtained by discontinuous centrifugation were transfused for active bleeding. The cytotoxic chemotherapy consisted of a combination of cytosine arabinoside (100 mg/m^2/day) for 5 days by continuous intravenous infusion, VP16–213 (60 mg/m^2/day) for the same 5 days by intravenous infusion over a 30-min period and adriamycin (40 mg/m^2) on day 6. Bone marrow aspiration and trephine biopsy were performed routinely 10 days after completing the course of treatment. If residual leukaemia was apparent in the marrow a second induction course was commenced using the same drug regimen. Patients shown to be aplastic received no further treatment until the bone marrow had regenerated and peripheral blood counts returned towards normal.

RESULTS

Twenty-eight of the 40 patients developed aplasia following a single course of therapy. Out of these 19 regenerated to complete remission (Group 1) and the other 9 died before regeneration had occurred (Group 2a). Of the remaining 12 patients, 5 had disease refractory to chemotherapy (Group 2b) and 7 died before the 10-day marrow examination and therefore could not be evaluated (Group 2c).

Group 1

Nineteen of the 40 patients (47.5%) entered complete remission having regenerated from their aplastic phase and all of these were under 50 yr of age. The average time to achieve an absolute neutrophil count of $1 \times 10^9/l$ or a platelet count of $100 \times 10^9/l$ following completion of the cytotoxic drugs was 16 days (range 6–29 days). Eighteen of these 19 (94.7%) developed sustained fever requiring antibiotics and white cell support. In 8 pyrexia was due to septicaemia with isolation of *Klebsiella* (4), *E. coli* (1), *staphylococcus* (2) and *streptococcus* (1). In the remaining 10 patients no organism was isolated from biological samples. However, 2 had a chest infiltrate on X-ray and 1 had unilateral hilar adenopathy with pleural effusion which was treated as tuberculosis.

Group 2a

Nine patients (22.5%) died following biopsy-proven aplasia. The mean time to death was 16 days (range 10–35 days).

Death was due to infection in 8 and to haemorrhage in 1. The mortality in this group was contributed to by the inclusion of 4 patients who were over the age of 50 yr and by 3 patients overtreated during the early stage of the study whilst experience was being accumulated with this treatment regimen.

Group 2b

Five patients (12.5%) had residual leukaemia after repeated courses of therapy in which 3 received 2 courses and 2 received 3 courses of the drugs. Four of the patients died and the fifth went on to successful syngeneic bone marrow transplantation. The mean time to death was 38 days (range 34–42 days). Our experience, therefore, suggests that if aplasia is not achieved after one course of treatment remission it is unlikely to be attained.

Group 2c

Seven deaths (17.5%) occurred before the tenth post-treatment day. Although blasts were no longer detectable in the peripheral blood, post mortem examinations were not obtained and accordingly these patient cannot be assigned to either of the above 2 sub groups.

DISCUSSION

The chemotherapy of acute non-lymphoblastic leukaemia remains largely empirical although it is based to some extent on data derived from animal models [8] and pharmacokinetic studies [9]. We have evaluated a 3-drug combination compromising the epipodophyllotoxin VP16–213, cytosine arabinoside and adriamycin. The VP 16–213 was incorporated because of its reported ability to potentiate the antitumour effects of cytosine [10]. Furthermore, we [11] and others [12] have shown it to be active against monocytic tumours and there is recent evidence [13] that a large population of these cells is present in the marrow of patients with acute myeloblastic leukaemia which is inapparent on examination of Romanovsky stained films. Since it has been suggested that cytosine arabinoside has the capacity to recruit cells into cycle [14] and lead to their synchronization [14] the adriamycin was administered at the completion of the cytosine infusion rather than at its commencement [15].

The time taken to achieve complete remission in adult acute non-lymphoblastic leukaemia varies widely and most investigators have reported the need to use more than one course of therapy. The MRC Working Party on Leukaemia in Adults [16] reported remission induction times for 5 different schedules to vary between 7 and 12 weeks. Glucksberg *et al.* [17] needed an average of 3 courses administered over 46 days to achieve complete remission in one group of patients and 1.4 courses given over 27 days in another. Wiernick [18] in 2 separate groups reported that at best 44% of patients achieving complete remission did so with a single course of therapy but the patient group as a whole required an average of 1.6 courses. Gale *et al.* [19] found that 35% of patients achieved complete remission with a single course with an average of 1.8 courses being needed.

In contrast, all our patients who achieved complete remission did so with a single course of chemotherapy with peripheral blood values rising in a time shorter than previously reported. It might have been anticipated that the short induction period would serve to reduce the incidence of infection. However, because this occurred early in the neutropenic phase no such benefit emerged and virtually all patients became infected. Nevertheless, this short period did have the advantage of decreasing the morbidity associated with infection since it is our experience and that of others [20] that final resolution of the infection only occurs once the bone marrow has regenerated.

The disparity between the number of patients who achieved aplasia and those going on to complete remission is accounted for by those (Group 2a) who died before their bone marrow had regenerated. Apart from the difference in age and the total white cell count at presentation (Table 1) these 2 groups did not differ in any other way particularly with respect to the incidence, duration and severity of infection. It is possible that the group of patients who died whilst aplastic may have survived had they been nursed in protected environments.

The poor response to treatment of individuals over the age of 50 has been noted [16] and was observed in our patients, reflecting the reduced tolerance of the elderly to the many consequences of septicaemia. An exception to this has been a recent report from Gale *et al.* [19]; here again, it is possible that access to protected environments may explain the difference.

We conclude that this 3-drug regimen has the advantage of rendering 70% of the patients aplastic with a single course of treatment with a short post-induction neutropenic phase. That some of these aplastic patients did not survive may reflect deficiencies inherent in treating adults with acute non-lymphoblastic leukaemia in general medical wards.

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Quelamycin, a New Derivative of Adriamycin with Several Possible Therapeutic Advantages*

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Abstract—*Quelamycin is triferric doxorubicine, a metallic derivative of adriamycin made by chelation of adriamycin at neutral pH with three ferric irons. This new derivative appears to maintain considerable antitumor activity while having less general, hematologic and cardiac toxicity than adriamycin. In addition, quelamycin is absorbed by the intestine.*

INTRODUCTION

IT WAS previously reported [1, 2] that the antitumour antibiotic, adriamycin, inhibits the activity of the cardiac enzyme Na–K dependent adenosine triphosphatase and also inhibits cellular ion transport. It was then claimed that these effects could be the cause for the cardiotoxicity of the drug [1, 2]. The effects of adriamycin on the ATPase and on ion transport were counteracted by the presence of calcium [2] and, as adriamycin is known to be a potent chelating agent [3], we made the hypothesis that metallic derivatives of adriamycin made by chelation would not be cardiotoxic. The present report presents a new metallic derivative of adriamycin, triferric doxorubicine, which has been made chelating adriamycin with three ferric irons at neutral pH. This derivative which has been named quelamycin for its use in clinical trials, appears to show considerable antitumor activity in experimental animal tumors while it has less general, haematological and cardiac toxicity. Quelamycin seems, furthermore, to present new properties concerning its permeability because it is active by oral administration. A previous report containing part of these data appeared in an abstract [4].

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MATERIALS AND METHODS

Isolation and assay of rabbit heart Na–K *ATPase*

The isolation and assay of rabbit heart Na–K ATPase was as described by Auditore and Murray [5] except that the freezing step was omitted. Four rabbit hearts were homogenized in 150 ml of a medium containing 0.25 M sucrose, 20 mM histidine, 5 mM EDTA and 0.1% deoxycholate (pH 7, 0°C). The homogenate was centrifuged at 9000 rev/min for 20 min. The supernatant free of mitochondria and nuclear debris was centrifuged at 52,000 ***g*** for 1 hr at 4°C. The supernatant was discarded and the pellet washed twice with 0.32 M sucrose plus 1 mM EDTA. This preparation had a mean Na–K-dependent ATPase activity of 0.2 μmole ATP/min/mg protein and amean sensitivity to ouabain of 50%. The enzyme activity was assayed after the incubation of the enzyme (0.1 mg/protein/ml) with the drug for 20 min in a shaking bath at 37°C. The incubation medium contained 80 mM imidazole, 3 mM Mg^+, 3 mM ATP, 140 mM Na^+ and 14 mM K^+ (pH 6.7). At the end of the incubation, samples were taken for the assay of inorganic phosphate. Phosphate was determined as described specifically for ATPase measurement by Post and Sen [6].

Incorporation of labelled thymidine

For incorporation studies of labelled thymidine, leukaemia L1210 cells were

harvested from the tumour samples by centrifugation at 600 *g* for 10 min at room temperature. The cell pellets were washed with 30 vol (30–60 ml) of a buffered electrolyte solution consisting of NaCl 117 mM, KCL 5.3 mM, NaH_2PO_4 1.1 mM, $NaHCO_3$ 26.2 mM, $CaCl_2$ 1.9 mM and $MgCl_2$ 1.0 mM (pH 7.45). Washed cells were resuspended in this solution (supplemented with 5 mM glucose) to a final concentration of 1.0×10^6 cells/ml. Samples of 1 ml of the cell suspension were preincubated at 37°C for 2 hr in the presence of the drug in a shaking bath. After this initial incubation, 1.0 μCi of thymidine-methyl^{3}H (2.0 Ci/mmole, New England Nuclear, Boston, Mass.) was added in 0.01 ml vol. The cell suspensions were incubated for 1 additional hr. After the second incubation, samples were chilled and the cells were immediately collected in 5 μm Millipore® filters and washed with 20 vol of ice cold 0.9% NaCl solution. The filters were placed in scintillation vials with 10 ml of aquasol scintillation mixture (New England Nuclear) and their radioactivities were measured in a Packard tri-carb model 3375 scintillation spectrophotometer. All experiments were done in duplicate with controls containing no antitumor drug.

Rat elctrocardiograms

Rat electrocardiograms were redorded with a Telco physiograph model B-52, essentially as described by the group of Zbinden [11]. Rats were unanesthetized but confined to a small animal holder. Needle electrode (26 gauge) was inserted into the skin using lead D as described by Grauwiler [7]. The paper speed was 10 cm/sec. Heart rate was monitored with a cardiotachometer coupled to the elctrocardiogram.

Animal treatments

The transplantation and evaluation of treatment of experimental animal tumours were made as described in the protocols used at the U.S. National Cancer Institute [8]. Tumours were obtained regularly from Dr. Abraham, Division of Cancer Treatment, U.S. National Cancer Institute, Bethesda. Lymphocytic leukaemia P388 was propagated in DBA/2 mice and transplanted for testing in BDF_1 mice (males, 20 ± 1.5 g), six animals per test group. The tumour transfer was done on day 7 by intraperitoneal injection of 0.1 ml diluted ascitic fluid containing 10^6 viable cells. Walker carcinosarcoma 256 in ascitic form was propagated in random bred albino Sprague–Dawley rats and transplanted for testing in random bred albino rats (Males, 60 ± 5 g), 6 animals per test group. The transfer was done on day 7 by intraperitoneal injection of 0.1 ml diluted ascitic fluid with 10^6 viable cells.

Ehrlich ascites tumour was propagated and transplanted for testing in Swiss mice (males, 20 ± 2 g), 6 animals per test group. The tumour transfer was done on day 9 by intraperitoneal injection of 0.1 ml diluted ascitic fluid with 10^6 viable cells. The tumours were treated intraperitoneally with adriamycin or quelamycin dissolved in water. The evaluation of antitumour activity was done by calculation of the median survival time from mortality distribution. Median Survival Time $= x + y/2$ where x is the earliest day when the cumulative number of deaths is $\geqq 3$ and y is the earliest day when the cumulative number of deaths is $\geqq 4$.

The LD_{50} of quelamycin and adriamycin was determined by intraperitoneal injection of a single dose with water as solvent in DBA/C57 F1 male mice (20 g of weight) with 7 days as observation period. A group of 20 mice was used for each dose.

RESULTS

Synthesis of quelamycin

For the synthesis of quelamycin (also called triferric doxorubicine or triferric adriamycin), it is necessary for each molecule of adriamycin in solution to chelate three atoms of ferric iron in the three bidentate ligands with high affinity at neutral pH. Thus, a good procedure for the synthesis is to join adriamycin in water at room temperature with three molar equivalents of ferric-chloride. Upon adding the iron, adriamycin changes its red colour to the black violet colour of ferric-adriamycin and the mixture is very acidic. The mixture is then neutralized slowly with NaOH to pH 7, under continuous agitation, to chelate the third iron with the amino sugar and the synthesis is completed. To avoid any excess of precipitated iron, it is convenient to filter the solution by a 0.2 μm Millipore® filter immediately upon neutralization. Quelamycin is only stable in solution for a few hours, because it tends to form polymers; thus, after filtration, it should be liophylized for storage as a dry powder which can be reconstituted by adding water. In the procedure of

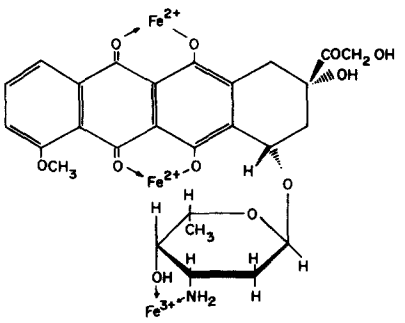

Fig. 1. Chemical structure of quelamycin.

liophylization, a quick freezing procedure is essential because polimerization is favoured by low temperature. The chemical structure of quelamycin is shown in Fig. 1. This structure was arrived at indirectly by determining the chelation sites of adriamycin at low iron concentration in neutral solutions of D_2O, using spectral analysis with an apparatus of nuclear magnetic resonance supplied with a computer averaging and summation system [10]. A maximum molecular weight of 700 was found by ultrafiltration indicating that over 99% of the chelate is in monomeric form [10]. As can be seen in Fig. 1, quelamycin has chelated the three irons in hexagonal and pentagonal rings of very high stability. Quelamycin remains undissociated in serum or tissue homogenates and is eliminated intact by urine after intravenous injection to the rabbit. Quelamycin is only slowly dissociated by heating in the presence of EDTA or oxalic acid.

Biochemical, biological and pharmacological effects of quelamycin

Figure 2 compares the effect of adriamycin and quelamycin on the activity of rabbit heart Na–K dependent ATPase. Adriamycin appears as a potent inhibitor of the enzyme while quelamycin does not have any effect. These experiments support the hypothesis that the counteracting effect of calcium in the effect of adriamycin in the enzyme [2] was due to its chelation by adriamycin. If the cardiotoxicity of adriamycin were due to its effects in the Na–K ATPase. quelamycin would behave as non-cardiotoxic.

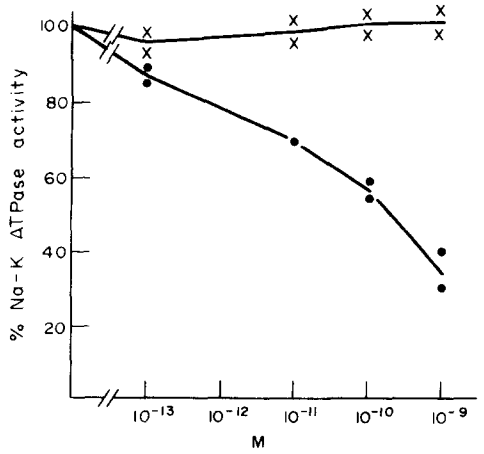

Fig. 2. Effects of increasing concentrations of quelamycin (crosses) and adriamycin (circles) on the activity of rabbit cardiac microsomal Na–K *ATPase.*

Figure 3 compares the average cardiac frequency and the QRS duration in the elctrocardiogram of groups of 5 rats treated with adriamycin and quelamycin. In the experiment of the lower part of the figure, the drugs were administered in a single intraperitoneal injection of 4 mg/kg daily,

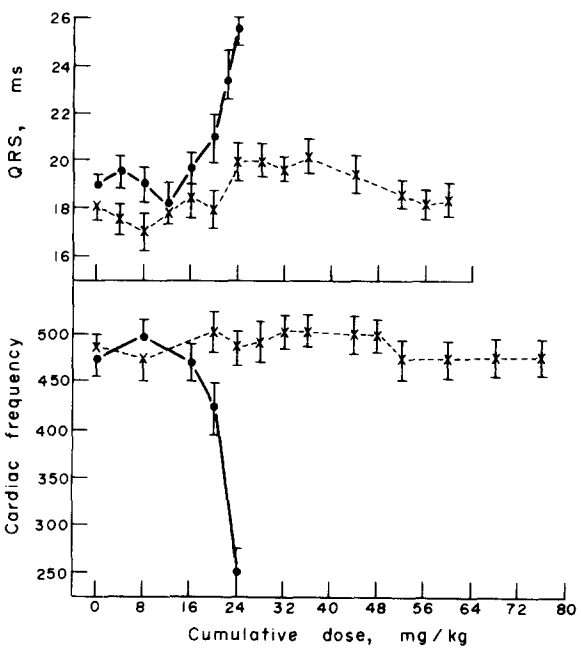

Fig. 3. Effects of cumulative doses of adriamycin (continuous line) and quelamycin (dotted line) on cardiac frequency and QRS widening in the rat electrocardiogram. Each point is the average value of 5 rats and the bar represents the standard error.

3 days per week. In the experiment of the upper part of the figure, the drugs were administered in a simple intraperitoneal injection of 2 mg/kg daily, 5 days a week for two weeks and then the dose was lowered to 1 mg/kg daily, 5 days a week. In both cases, when a cumulative dose of 24 mg/kg of adriamycin per rat was reached, the rats died, showing a great QRS widening and an intense bradicardia. In the groups treated with quelamycin, the rats reached much higher cumulative doses and survived without showing signs of electrocardiographic alteration. The observation was

continued for four months and no delayed cardiotoxocity appeared. In the group of rats treated with quelamycin, a QRS widening was found occasionally in individual rats. This widening later disappeared after a few days without further increasing when increasing the cumulative dose.

Aside from a decreased cardiotoxicity, quelamycin showed less general, haematological and digestive toxicity. Figure 4 (left graph) shows the mortality distribution of male mice of 20 g of weight (DBA/C57 F1 strain) after 7 days of a single intraperitoneal injection of adriamycin or quelamycin at different doses. Quelamycin has a LD_{50} of 45 mg/kg and adriamycin has a LD_{50} of 15 mg/kg. The right graph of Fig. 4 shows the variation in leucocyte count in dogs after the injection of 2 mg/kg of adriamycin or quelamycin. Each point is the average of the counts in two dogs. Quelamycin depresses the leucocyte count much less than does adriamycin.

considerable activity in the three tumour systems and quelamycin, due to its lower toxicity, could be used at higher doses.

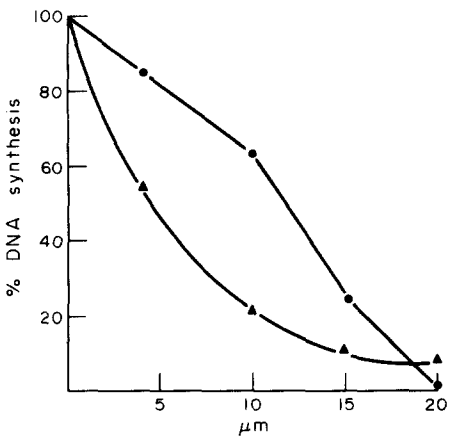

Fig. 5. Inhibition of DNA synthesis by increasing concentrations of quelamycin (circles) and adriamycin (triangles) as measured by uptake of tritiated thymidine in leukaemia 1210 cells. Each experimental point shows the average of three determinations.

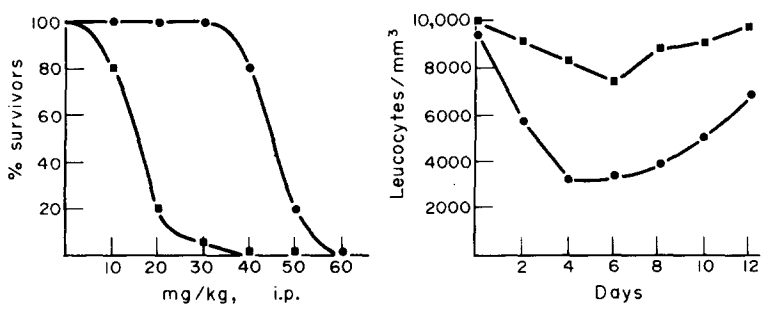

Fig. 4. (Left graph) Mortality distribution in mice after seven days of a single intraperitoneal injection of adriamycin (squares) or quelamycin (circles) at the indicated doses. (Right graph) Average leucocyte counts in the peripheral blood of two dogs in the days following a single intravenous injection of 2 mg/kg *of adriamycin (circles) or* 2 mg/kg quelamycin (squares).

While showing less toxicity, quelamycin maintained antitumour effect. Figure 5 shows the effect of adriamycin and quelamycin on the DNA synthesis of leukaemia L1210 cells as measured by uptake of tritiated thymidine. Both compounds appear as good inhibitors of DNA synthesis with similar potency. Table 1 illustrates the antitumour activity of quelamycin and adriamycin with a representative experiment in Ehrlich ascites tumours, Walker carcinosarcoma tumours and leukaemia P-388 tumours. Each experimental group consisted of six animals and the drugs were injected intraperitoneally at the indicated doses on day 1, 5 and 9 (P-388) or on day 1, 2, 3, 4 and 5 (Walker carcinosarcoma and Ehrlich ascites tumour) after transplantation. Both compounds showed

The antitumour activity of quelamycin and adriamycin varied considerably from experiment to experiment and this variation, in the absence of a large statistical study, precluded any conclusion about which drug shows more potent antitumour activity in each tumour. Figure 6 shows the leucocyte counts in peripheral blood in three cycles of administration of quelamycin in two dogs. The first and third cycles were of 4 mg quelamycin/kg using enterric-coated pills, and in the second cycle the same dose was administered by intravenous route. It can be observed that the bone marrow suppression is more complete with the intravenous dose but that there is considerable bone marrow depression activity with the pills. The pills were manufactured using

Table 1. Median survival time and survivors in ascites tumours treated with adriamycin and quelamycin

Ascitic lymphocytic leukaemia P-388				Median survival time (days) and survivors					
Dose mg/kg days 1, 5 and 9	0	0.5	1	2	4	8	12	24	36
Adriamycin	9 (0)	16 (0)	17 (0)	20 (0)	22 (0)	12 (0)	8 (0)	—	—
Quelamycin	9 (0)	15 (0)	18 (0)	19 (0)	21 (0)	20 (0)	22 (0)	22 (0)	16 (0)
Rat walker carcinosarcoma, ascites form				Median survival time (days) and survivors					
Dose mg/kg days 1, 2, 3, 4 and 5	0	2.5	5	10					
Adriamycin	7.5 (0)	50 (0)	6 (0)	—					
Quelamycin	7.5 (0)	59 (5)	50 (4)	35 (2)					
Ehrlich ascites tumours				Median survival time (days) and survivors					
Dose mg/kg days 1, 2, 3, 4 and 5	0	2.5	5	10					
Adriamycin	17 (0)	25 (0)	7 (0)	—					
Quelamycin	17 (0)	34 (0)	36 (1)	15 (0)					

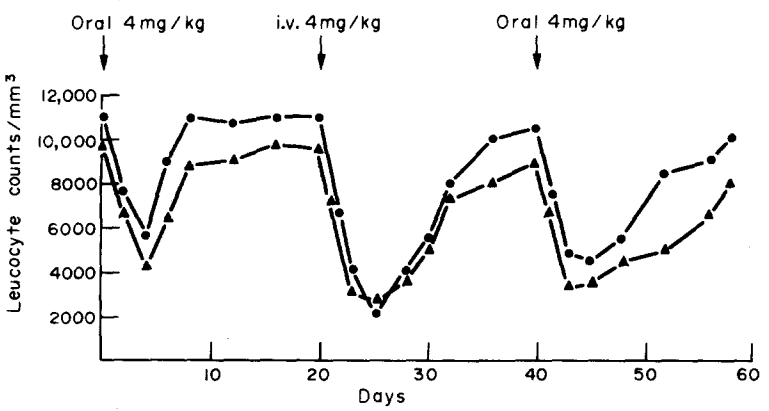

Fig. 6. Peripheral blood leucocyte counts/mm³ in two dogs treated with quelamycin with oral or intravenous doses of 4 mg/kg.

liophylized powder of quelamycin with 1% mannitol as support, using magnesium stearate and carboxymethyl cellulose as excipient and received a gastro resistant coat of acetylftalate of cellulose and dimethylftalate to pass through the stomach unaltered.

DISCUSSION

From our results, it appears that quelamycin is a derivative of adriamycin which maintains considerable antitumour effect while having less general, haematologic and cardiac toxicity. Our results in experimental tumours were obtained in a limited series of tumours and with a limited number of doses. To ascertain that quelamycin has equal antitumour potency to adriamycin milligram to milligram, it would be necessary to carry out larger studies. It is possible that, due to possible different permeability properties of quelamycin, the drug would show different activity in different tumours.

Quelamycin seems well tolerated over large cumulative doses from the electrocardiographic point of view. However, a transient QRS increase was noted in some animals. It is possible that Quelamycin could have been deprived of direct effects on cardiac contractility through lacking effect on the Na–K ATPase but would still have cardioactivity through neurological mechan-

isms at high doses, as is the case with other glycosides [12–14]. Nevertheless, to ascertain a lack of cardiotoxicity with quelamycin, a morphological study in the rabbit model cardiomyopathy [15] would be necessary; such studies are presently under way [16].

Quelamycin is active when administered orally but the haematological effect is less intense than with intravenous administration. The oral absorption could indicate permeability characteristics which would be suitable to cross the blood–brain barrier. Preliminary experiments have shown that quelamycin, when administered intravenously at high doses, crosses the blood–brain barrier in the rabbit but not in the mouse.

The apparent advantages of quelamycin over its parent compound, have prompted clinical studies in man. Presently, from a phase I clinical study carried out at the five major Madrid hospitals, it has been concluded that quelamycin had considerable antitumour activity and low toxicity. However, to ascertain the possible activity of quelamycin, the results of the ongoing phase II trial should first be completed.

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Combination Chemotherapy of Malignant Glioma. Effect of Postoperative Treatment with CCNU, Vincristine, Amethopterine and Procarbazine

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Abstract—*A combination chemotherapy consisting of CCNU, vincristine, amethopterine and procarbazine (COMP-therapy) was studied in the postoperative treatment of malignant gliomas. Fifteen-day COMP-therapy series given* 10–20 days *after surgery and repeated at regular intervals increased the median recurrence-free interval to* 8.9 *months and the median survival time to* 10.7 *months. Both prolongations were significant when compared to the results in a control group not receiving postoperative chemotherapy (median recurrence-free interval:* 2.6 *months, median survival time:* 6.2 *months), in a group receiving postoperative CCNU-treatment* (3.5 *and* 6.5 *months, respectively), and to a group of patients treated with postoperative polychemotherapy* (4.7 *and* 8.0 *months, respectively). The only problematic side effect of COMP-therapy was neuropathy resulting from toxic vincristine-dosage.*

While nitrosoureas alone were not able to affect the length and the quality of survival, their combination with cell-cycle specific and other cell-cycle non-specific drugs improved the results in the therapy of primary malignant brain tumors.

INTRODUCTION

IN THE search for a more effective treatment of malignant glioma (glioblastoma, astrocytoma grades III–IV) combined modalities of surgery, radiotherapy and/or chemotherapy have been shown to be superior to surgical removal of the tumor alone. The median survival time for glioma patients with surgery only was between 4 and 6 months [1, 2]; extensive surgery and radiotherapy were able to prolong the median survival time [1–7], although this was not observed by all authors [8, 9]. Whereas some studies [1, 6, 9] showed that the addition of chemotherapeutic agents to the postoperative radiation therapy improves survival rates, others [2, 5, 7] did not find them to be significantly effective. Survival rates with postoperative chemotherapy alone were below those obtained with radiation therapy or combination of both [1, 2, 6, 7], but a high percentage of recurrent gliomas responded to a single drug [10–15, reviews in 16, 17] or combination chemotherapy [18–20]. The poor results with chemotherapy of gliomas were attributed to limited concentrations of drugs in the tumor cells due to the blood–brain-barrier [16, 21–23], a conclusion which has been recently questioned, however [24]. It has been shown previously [25] that a combination chemotherapy consisting of drugs not selected according to their ability to penetrate the blood–brain-barrier was superior to a monotherapy applying a single lipid soluble nitrosourea-derivative. This indicates that factors other than lipid solubility and the drug ability to penetrate into the normal brain must also be considered in the design of chemotherapeutic protocols. Such potentially effective factors include the route of administration and the combination of drugs. By application of several drugs in appropriate schedules sufficient amounts of effective agents might accumulate within the tumor cells. It was the purpose of this investigation to prove the efficiency of a combination of four antineoplastic drugs in postoperative treatment of malignant gliomas and to compare the results with those obtained under other protocols.

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MATERIALS AND METHODS

Patients

The following drugs were selected for the combination chemotherapy (COMP) because they had previously proved to be effective in brain tumors (Table 1): 1-(2-chloroethyl)-3 cyclohexyl-1-nitrosourea (CCNU) is a cell-cycle non-specific lipophilic drug which readily penetrates into the brain tissue [26]. Its effectiveness on brain tumors has been reported repeatedly [13, 14, 27, 28]. Encouraging results with brain tumors have been obtained [15] with procarbazine, another cell-cycle non-specific compound which crosses the blood–brain-barrier [29].

Table 1. *COMP-protocol*

CCNU	100 mg/m^2 p.o.	1 day
Vincristine	1.4 mg/m^2 i.v.	1 + 8 day
Amethopterine	10 mg/m^2 i.m.	3, 7, 10 + 14 day
Procarbazine	100 mg/m^2 i.v.	2–15 day

Vincristine (VCR: Oncovin®), a cell-cycle specific drug which inhibits the cell division at metaphase, has been effective in some studies [10, 30, 31]; however, other authors do not consider it of value for the glioma therapy [32, 33]. Amethopterine (methotrexate), an S-phase specific drug, is taken up into neoplastic glial cells [34], but does not achieve high concentrations in normal brain tissue [35]. When given as intracarotid infusion [36], intrathecally [37] or intraventricularly [38], some effect upon gliomas could be seen.

The COMP-protocol, a combination chemotherapy employing CCNU, vincristine, methotrexate and procarbazine, was applied in 15-day series: the first series was initiated 10–20 days after surgery, the second and third in 6-week intervals thereafter, and the following cycles then were administered in 3-month intervals until recurrence of the tumor or death of the patient. When severe toxic side effects appeared treatment was modified or stopped. All patients received steroids (methyl-prednisolone, 20 mg/day) during the chemotherapeutic series and afterwards until the white blood count was normalized. When necessary, gastrointestinal side effects were treated, antibiotics were given and fluids and electrolytes were balanced.

Twenty-two consecutive patients suffering from malignant glioma (glioblastoma, astrocytoma grades III and IV, according to Kernohan *et al.* [39], received postoperative chemotherapy under the COMP-protocol. In all these cases as much as possible of the tumorous tissue was resected: specimens were obtained for histological and cytological examination. With a few exceptions autopsies were performed. If the biopsy-diagnosis was not confirmed by the postmortem histological examination, patients were excluded from the study. The patients underwent general and neurologic examinations before and regularly during the course of the COMP-protocol. EEG, brain scintigraphy, electroneurographic investigations and blood tests were performed at regular intervals. If necessary, cerebral angiography or computer tomography were undertaken.

In order to evaluate the effect of the COMP-therapy two criteria were used: (1) the survival time, the most clearly defined and most frequently used parameter; and (2) the time interval between surgery and recurrence of the tumor, which approximates the duration of "good" quality of life. This progression free interval was determined from clinical symptoms and/or occurrence of localized pathological isotope uptake in the scintigrams. Both criteria were compared with the results in groups of unselected patients treated previously under other protocols [25]. Those 55 patients also suffered from malignant gliomas diagnosed by the same neuropathologist without knowledge of the mode of further treatment. Sixteen patients who received conventional therapy—i.e. surgery only, or, in some cases, surgery plus irradiation—comprised the control group. Twenty four patients received 100 mg/m^2 CCNU in 6-week intervals following the operation. A third group of 16 patients were administered postoperatively a polychemotherapy (PChTh) described by Israel *et al.* [40]; the following cytotoxic agents were given in 1–4 consecutive 7-day cycles: procarbazine (125 mg i.v.) and 6-mercaptopurine (50 mg p.o.) daily; cyclophosphamide (300–500 mg i.v. infusion) on day 1, 3 and 5; 5-fluorouracil (250 mg i.v. infusion) on days 2, 4 and 6; amethopterine (5 mg i.m.) on day 1 and 4; vinblastine (5 mg i.v.) on day 7. These and the other patients received steroids (20 mg methyl-prednisolone) before and several weeks after surgery; steroids were also given intermittently to all patients when necessary during the latter course of their disease. In no case, neither under the COMP- nor under the other protocols, were steroids administered for a prolonged period of time in high dosages.

Table 2. Recurrence-free interval and survival time in months (median and range) of patients receiving postoperative COMP-therapy compared to patients under other protocols. Significant H-values of variance analysis are given ($P<0.01$)

	N	Age (yr)	Recurrence-free interval (months) median	range	Survival time (months) median	range
Controls	16	22–66	2.6	1.0– 9.0	6.2	1.0–11.4
CCNU	23	35–76	3.5	1.0–15.7	6.5	1.5–18.5
PChTh	16	18–69	4.7	2.5– 9.8	8.0	3.3–18.2
COMP	22	31–64	8.9	3.0–26.0	10.7	3.5–27.8
$\hat{H}$			15.96		12.84	

Survival times and recurrence-free intervals achieved under the different protocols were statistically evaluated by variance analysis using ranks (H-test of Kruskal and Wallis [41]). The results of the COMP-Therapy were compared to those of the control and of the CCNU group by the Wilcoxon rank sum test [42]; if necessary, the version of Gehan [43] was used.

RESULTS

Survival time

As shown in Table 2, the patients receiving postoperative COMP-therapy survived surgery by 3.5–27.8 months (median survival time 10.7 months). Six of these patients are still alive 9.5–27.5 months after surgery. The survival time is longer than the

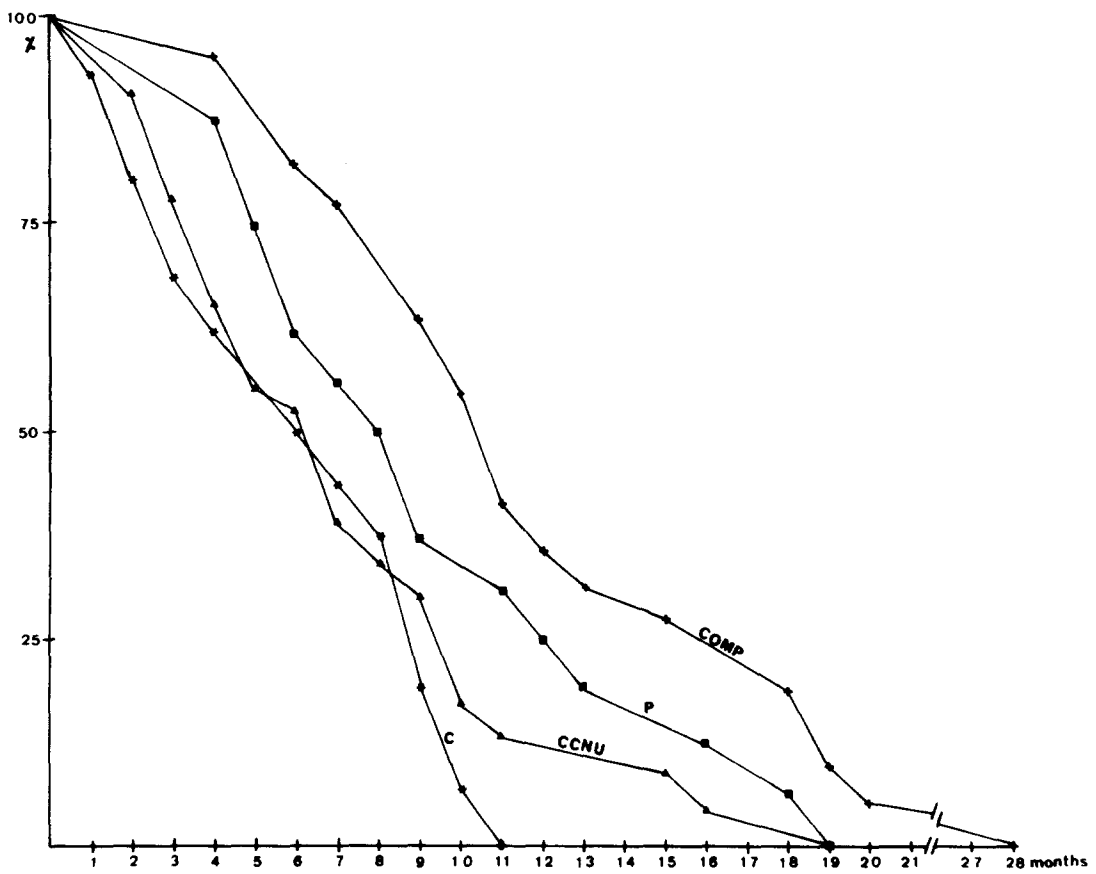

Fig. 1. Survival curves of patients under different protocols: C: control group, 16 patients; CCNU: 100 mg/m² every 6 weeks, 23 patients; P: polychemotherapy, 16 patients; COMP: COMP-therapy, 22 patients.

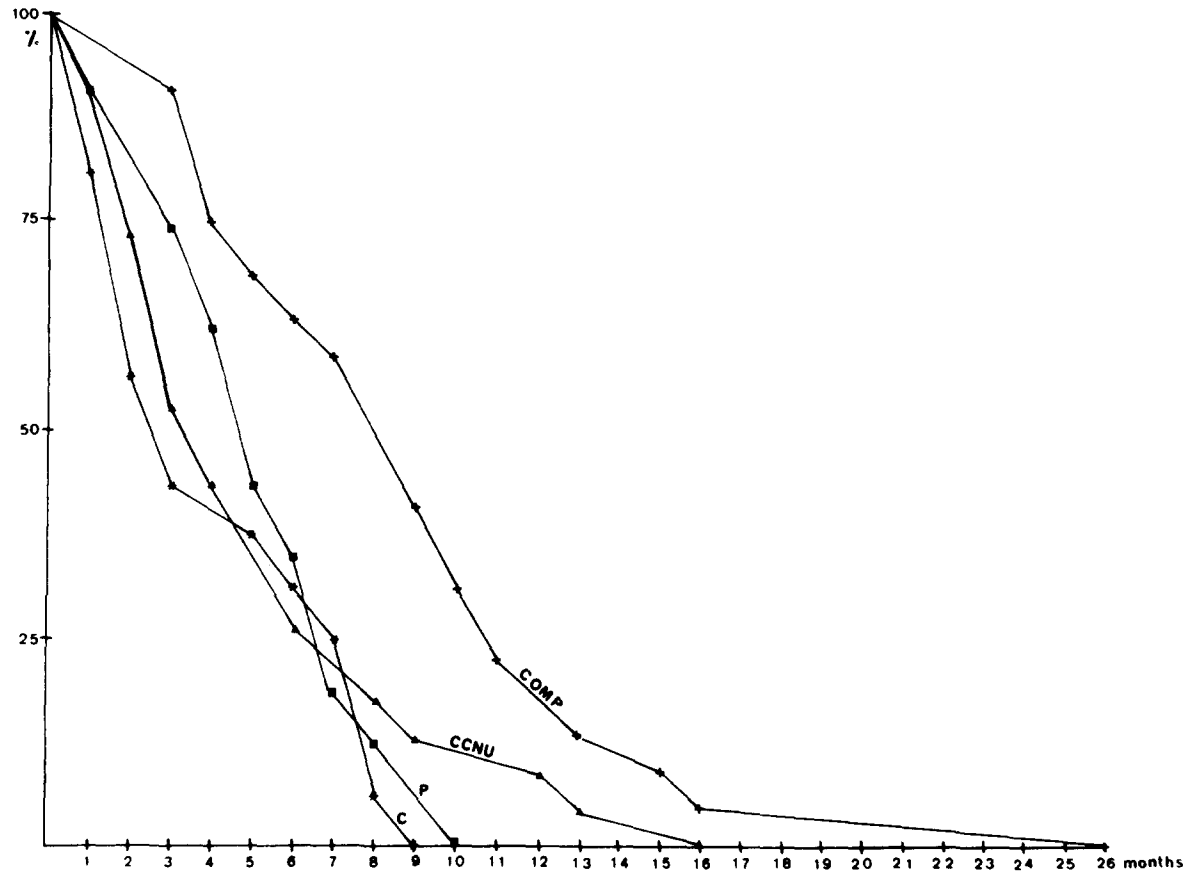

Fig. 2. Curves of recurrence-free intervals under different protocols. C: control group, 16 patients; CCNU: 100 mg/m² CCNU every 6 weeks, 23 patients; P: polychemotherapy, 16 patients; COMP: COMP-therapy, 22 patients.

survival times observed with the previously used protocols; this improvement shows up in the significant H-value from the variance analysis and in the survival curves of the patients (Fig. 1). The subsequent comparison of survival times in individual groups using Gehan's modification of the Wilcoxon-test showed significant differences of COMP-therapy to the control group ($z=3.2$, $P<0.01$) and to the group receiving CCNU only ($z=3.52$, $P<0.01$).

Recurrence-free interval

The recurrence-free intervals observed under COMP-therapy are also given in Table 2: they were between 3.0 and 26.0 months with a median of 8.9 months. Two patients included in the group were not suffering from recurrence 10.0 and 10.5 months after surgical removal of their glioma. Again, the recurrence-free intervals were significantly longer with the COMP-therapy than in the other protocols, proved by the high H-value and demonstrated in the curves of time intervals to recurrence (Fig. 2). Comparison of the groups showed significant differences of the COMP group to the control and to the CCNU group ($P<0.01$). The quality of the recurrence-free interval, i.e. the severity of neurologic deficits, was not used as a criterion for efficacy of the treatment, because the neurologic defects depend on the brain damage done by the tumor and surgery. Glioma patients with and without postoperative treatment reach their best clinical condition soon after surgery; its prolongation is the aim of the postoperative treatment.

Determination of recurrences

The patients underwent regular clinical neurological examinations; EEG and brain scintigraphy were performed at 4–6 weeks intervals. If neurologic symptoms developed or deteriorated, the patients were investigated immediately. In 8 patients brain scintigraphy confirmed recurrence of the tumor immediately after it was clinically suspected. In 2 patients a recurrence was indicated by clinical signs, but brain scintigraphy did not yet show a clear-cut localized isotope

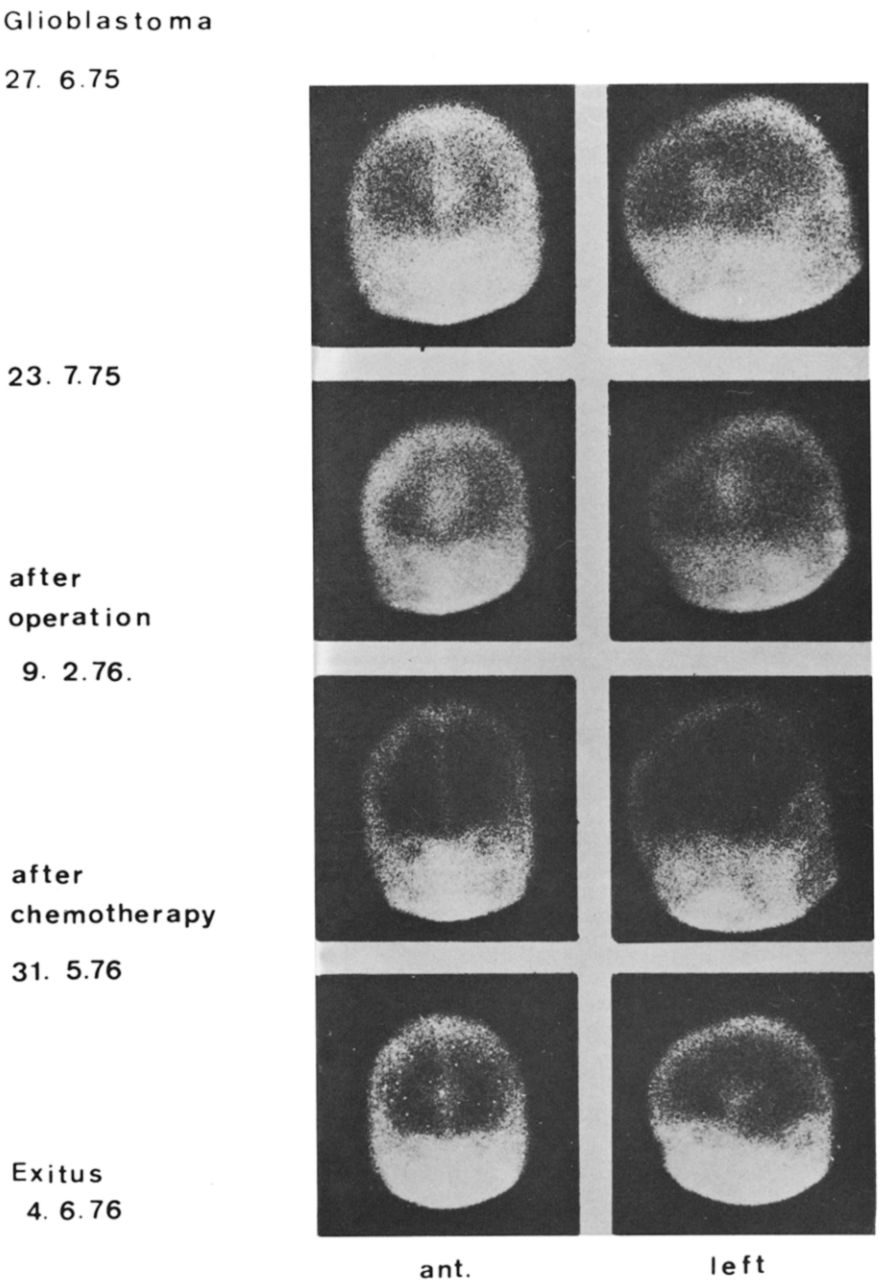

Fig. 3. Scintiphotograms of a patient suffering from butterfly-like glioblastoma. The tumor mass remaining after surgery disappeared during COMP-therapy, a recurrence showed up 3 months later and the patient died thereafter.

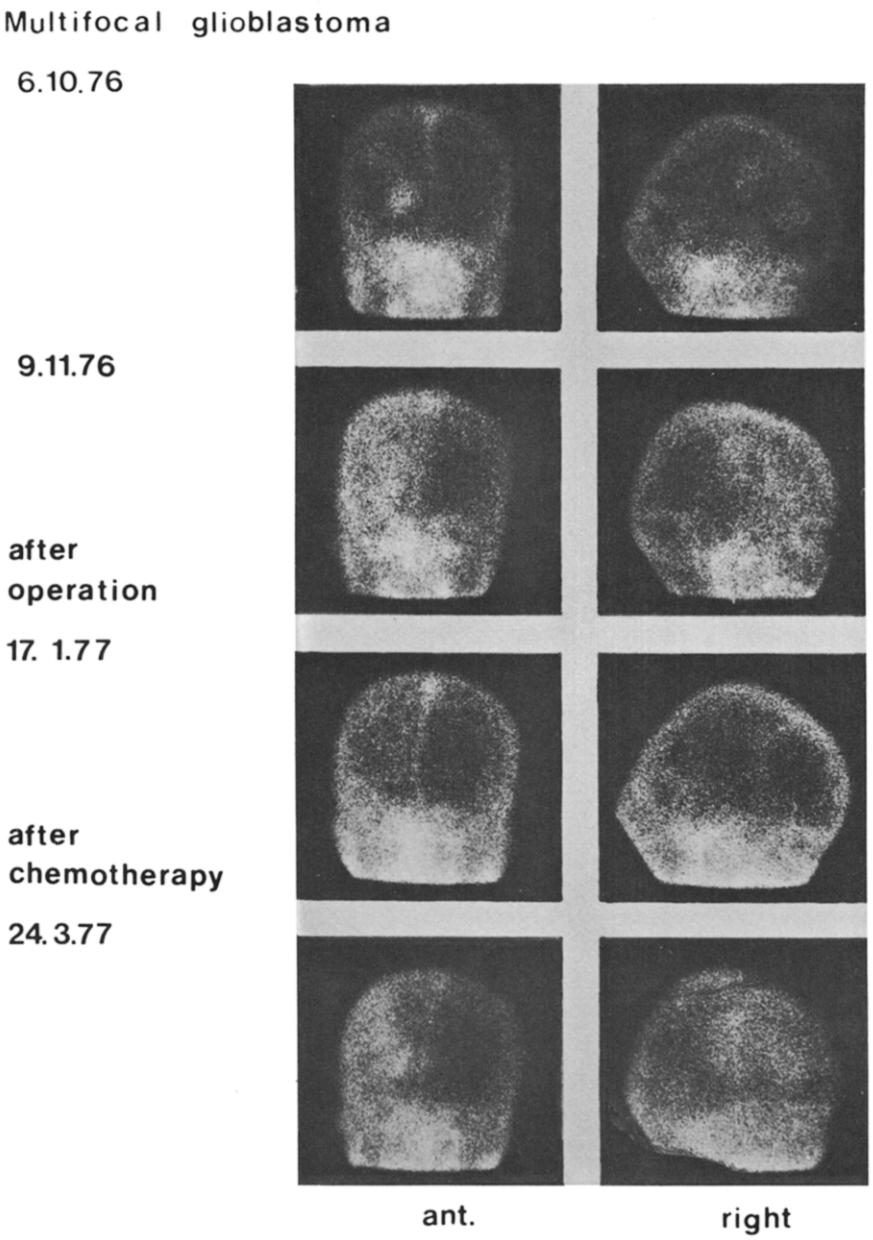

Fig. 4. Scintiphotograms of a patient with multifocal glioma. The frontal center was removed, the parieto-central tumor mass decreased during COMP-therapy. Recurrence occurred 3 months later.

uptake; scintigraphy was positive 2–3 weeks later. In 10 cases brain scintigraphy was positive before clinical signs indicated the recurrence: localized isotope uptake developed between 1 and 14 weeks before the condition of the patient deteriorated. Recurrence-free interval was defined as the time between surgery and the first evidence of a new or increased tumor-mass indicated by clinical deterioration or brain scintigraphy.

Brain scintigraphy could show the effect of therapy in those cases in which removal of the tumor was incomplete or, as in two cases with multifocal glioma, only one tumor could be excised. In such cases the region of increased isotope uptake caused by remaining tumorous tissue (Fig. 3) or by the second glioma center (Fig. 4) decreased its extension and intensity or disappeared completely. However, the effect of the therapy on these gliomas visualized by scintigraphy and expressed in clinical improvement did not last more than a few months.

Electroencephalography was not as effective in indicating recurrences; in 9 of 11 cases a focus was found shortly after the occurence of clinical or scintigraphical signs. In 2 patients EEG did not change markedly when tumor progression was evident from other sources.

Tumor recurrence was treated by surgery in 10 patients receiving postoperative COMP-therapy. The clinical condition of these patients was improved under further chemotherapy in 3 cases, the improvement lasting for 4 months in two cases and 6 months in one. In the other 7 cases tumor progression could not be delayed and these patients died soon thereafter. In the two cases with multifocal gliomas the second tumors were removed when they could not be further influenced by chemotherapy. In these cases as well surgical intervention had no effect on the clinical course.

Side effects

Gastrointestinal toxicity was the most consistently observed side effect. Gastrointestinal irritations—nausea and vomiting—were observed in more patients during the first few days of the COMP series; they could be suppressed by antiemetic drugs and usually disappeared after the 3rd day. All the patients complained of loss of appetite during the series.

Hematopoietic toxicity was frequently encountered: in most patients white blood cell counts lower than $3000/mm^3$ were seen after the individual COMP series, but the white blood cell counts usually recovered to above 4000 before the next series. In 7 instances white blood cells fell below $2000/mm^2$ with a specific depression of granulocytes (40% or below); as in the other cases bone marrow showed only slight toxic alterations and the counts improved with prolonged steroid therapy. Platelet depression was observed in most patients after the COMP series, but only in 3 instances were counts below $65{,}000/mm^3$. Neither infections nor bleeding were seen as a result of leukopenia or thrombocytopenia. Only a single case of transient depression of erythrocytes occurred. The transient hematopoietic toxicity was not considered as a contraindication to continued chemotherapy; in some instances the next series was postponed by 1–2 weeks until the counts reached normal values.

Increased serum enzymes indicating liver toxicity (alkaline phosphatase, transaminases) were observed in 10 cases, but the values usually returned to normal before the next series was started. Renal toxicity with increase in serum creatinine and urea for a few weeks was seen in only one case.

Neurotoxicity was the most severe side effect. All patients developed symptoms of peripheral neuropathy with loss of tendon reflexes, distal paresthesia, and, in few instances, transitory distal limb weakness; these symptoms were dependent upon the total dosage of VCR received. The neuropathy was objectified by electrophysiological investigation. Maximal motor nerve conduction velocity (NCV) of the right peroneal nerve and compound action potential (CAP) of the short digital extensor were measured: pathologic NCV values were recorded (Fig. 5) in 5 of 15 patients after 2–6 mg VCR; in 6 of 12 patients after 7–12 mg VCR; and in 10 of 12 patients after 13 mg or more VCR. In one case excitability of the peroneal nerve was completely lost after 16 mg VCR. The mean conduction velocity in the patients who received the respective total dosages of VCR were: 44 m/sec after 2–6 mg VCR, 41 m/sec after 7–12 mg VCR, 40 m/sec after 13–18 mg VCR, and 39 m/sec after more than 18 mg VCR. CAP was within the pathology range in 2 of 14 patients after 2–6 mg VCR, in 7 of 14 patients after 7–12 mg, in 7 of 11 patients after 13–18 mg and in 4 of 5 cases after more than 18 mg VCR. The mean values

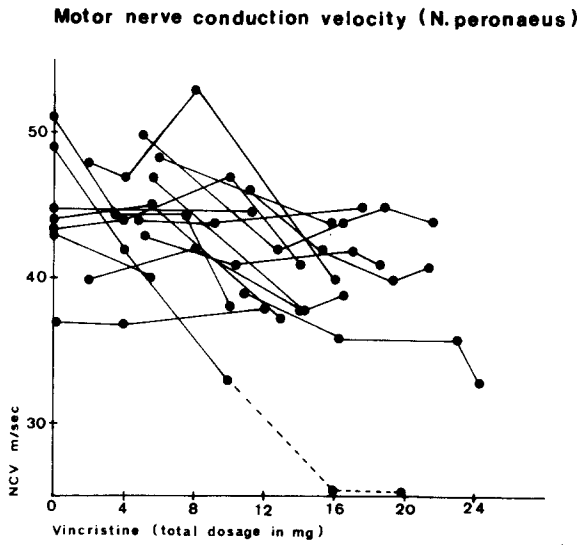

Fig. 5. Change of motor nerve conduction velocity (m/sec) *of the peroneal nerve of individual patients with increasing total vincristine dosage.*

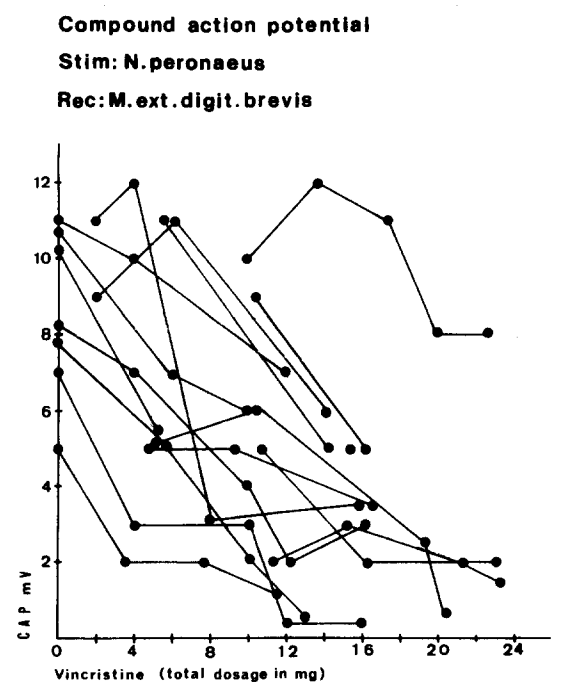

Fig. 6. Change of compound action potential (mV) *of M. ext. digit. brevis after peroneal nerve stimulation in individual patients with increasing total vincristine dosage.*

of the respective groups were 7.5 mV after 2–6 mg VCR, 4.6 mV after 7–12 mg VCR, 3.9 mV after 13–18 mg and 2.5 mV after more than 18 mg. The marked decrease of CAP compared to the slight alteration of NCV indicates a primary axonal neuropathy. In view of the electrophysiological alterations patients were not given more than 2 mg or half the original dosage, i.e. 0.7 mg/m^2 during the later COMP-series. By means of these controls and the consequent decrease in dosage, persistent clinically established neuropathies could be avoided.

DISCUSSION

After extensive experience with single-agent chemotherapy, especially for recurrent brain tumors [17], combined drug schedules and prophylactic treatment regimens must be tried to achieve substantial prolongation of survival time and improvement of quality of life during this time, as is now the case in the treatment of other malignancies [44, 45]. Due to the peculiarities of gliomas with respect to growth fraction and cell kinetics [46] the drug combinations must include both cell-cycle non-specific and cell-cycle specific agents in order to achieve a high tumor cell kill. Since the growth fraction increases secondarily to a decrease in tumor size after effective treatment, retreatment is necessary after a 3–4 week interval. Furthermore, removal of substantial parts of the glioma forces the remaining tumor cells into an active growth phase during which they are more susceptible to chemotherapy [47]. Therefore, chemotherapy was initiated in our cases 10–20 days after surgery, at a time when the tumor mass is minimal and the blood–brain-barrier is still disrupted. In consideration of tumor repopulation, the chemotherapeutic series were repeated twice after therapy-free intervals of 4 weeks, and, later, to avoid severe toxic side effects, after 10–11 week pauses. This regimen of therapy did not permit evaluation of the effect of chemotherapy by dividing the patients into "responders", "probable responders" and "non-responders" and calculating response rates [48]. The prerequisite of such an analysis, the continuous deterioration due to tumor regrowth, is not fulfilled. The effect of a therapy aimed at prevention of tumor regrowth can only be evaluated by comparing recurrence-free intervals and durations of survival with the same criteria achieved by different kinds of treatment.

In prospective randomized, concurrent, controlled clinical trials some regimens including chemotherapeutic agents [5–7] were tested.

Results obtained in consecutive and not concurrent studies are not devaluated by the method of their accumulation [49] as long as the patients in the different groups belong to the same population and are clinically and histologically examined by the same investigators, and as long as data from "historical" controls used agree with

those in the literature. In our study, the results with the COMP-therapy are compared to groups of patients investigated in the past: survival time and recurrence-free interval of the control group are in the range of those reported in the literature and of those obtained in a large follow-up study of glioblastoma patients surgically treated by the same neurosurgical team as ours [50]. Patients with and without postoperative radiotherapy were comprised in one group because of statistical reasons. This procedure was justified by comparable recurrence-free intervals and survival times of these patients. That the postoperative radiotherapy used in Vienna in the past could not improve survival time significantly was shown in the large groups of Grunert *et al.* [50]. The results obtained with post-operative CCNU monotherapy were not different from the controls, which is in accordance with previous reports [1, 2, 6, 7]. However, the polychemotherapy described by Israel *et al.* [40] slightly increased recurrence-free interval and duration of survival [25]. Compared to those protocols COMP-therapy had a significant effect not only on survival time, but also on the recurrence-free interval and, thereby, significantly prolonged the useful life-span for these patients. This prolongation of "good" quality of life, which was not combined to a prolongation of the time interval between recurrence of the tumor and the death of the patient, could not be due to the effect of steroids: patients in the other groups also received steroids; and a steroid effect on long-term survival could not be demonstrated with different administration schedules [51]. The significant improvement of quality and length of survival under COMP-protocol as compared to CCNU treatment suggests that other chemotherapeutic agents have to be added to nitrosoureas. The same was also indicated by the comparable results obtained with a combination of postoperative radiotherapy and nitrosourea-chemotherapy [1, 6, 9].

As in previous studies [48, 52] brain scintigraphy was the most reliable criterion in the detection of recurrences: by this method new tumor masses could be demonstrated in 10 cases before clinical signs indicated the recurrence, whereas clinical symptoms suggested a new tumor only in two cases with negative scintigrams. Computer tomography, which is also a reliable means for controlling therapeutic effects [53] has only been available for $1\frac{1}{2}$ yr and could not, therefore, be systematically evaluated for comparison to the previous protocols.

The toxic side effects of the COMP-therapy were less severe than with PChTh, during which strong bone marrow depression observed in a few cases necessitated the interruption of the treatment. There was no case in which the COMP-protocol had to be discontinued due to hematopoietic complications. The only drug the dosage of which had to be reduced in the latter course of therapy was vincristine: as described previously [32, 54–56] the toxic neuropathy of axonal origin is dependent on the total dosage of vincristine, but severe clinical symptoms may be avoided or are reversible when the drug is not administered further or when lower single doses are given. While vincristine-neuropathy was not a severe problem in our cases, it might be a limiting factor with substantial increase in the survival time.

In our experience COMP-therapy is the first postoperative treatment of glioma patients leading to a substantial improvement of good quality life. The still unsatisfactory results might be improved by changing the constituents of the combination, the dosage and the timing of administration of the individual drugs, and by combining chemotherapy with postoperative radiotherapy.

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Tumour Immunoprophylaxis Exerted by the Antimicrococcus Immunity. I. Influence on the Proliferation of Murine Leukaemic L1210 Cells*

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Abstract—*An immunization schedule that leads to the production of antimicrococcus antibodies of restricted electrophoretic heterogeneity in (female BALB/c × male DBA/2) F_1 mice (CDF_1) is described and commented on.*

Although mouse antimicrococcus sera agglutinate cultured L1210 cells in vitro, *micrococcus vaccinated mice never showed any anti-leukaemia immunoprotection. However, vaccinated mice, given booster doses of* 1 mg *heat-killed* Micrococcus lysodeikticus *on* days 4, 8 *and* 12 *after a tumour challenge of* 10^4 *cells, were considerably protected* ($P<0.001$) *and showed a* 92% *increase in mean life span over untreated controls, with* 57% *of long-term survivors, whereas unvaccinated mice, given* 1 *mg micrococcus injections on* days 4, 8 *and* 12, *showed a* 72% *increase in mean life span* ($P<0.001$), *but no long-term survivors were recorded.*

Our results underline the idea that, after establishment of tumour growth, the humoral antitumour immunity requires an adequate trigger of immunocompetent effector cells to be operationally effective.

INTRODUCTION

IN MAMMALS, the immune response to glycoprotein is predominantly directed at protein antigenic epitopes and not at the carbohydrate moiety [1].

Because of the similarity of the carbohydrate moiety of the serum glycoproteins and of the glycoproteins that function as cell surface receptors, it is likely that animals have acquired selective tolerance and recognize those polysaccharides as "self" antigens [2, 3]. However, when animals are infected or vaccinated with Gram-positive *Streptococci* [4], *Pneumococci* [5] or non-pathogenic *Micrococci* [6] that carry an analogous carbohydrate as a part of the bacterial cell wall, the elicited anti-carbohydrate immune response is abundant and often of restricted electrophoretic and structural heterogeneity (homogenous antibodies), a characteristic in common with the well studied myeloma proteins [7].

Rabbit antibodies against *Micrococcus lysodeikticus* (ATCC 4698) are of high titer (up to 40 mg/ml serum) [6] and are directed [8] either against the bacterium specific carbohydrate, called Perkins' antigen (a copolymer of glucose and *N*-acetyl-D-mannosaminuronic acid), or against the bacterial peptidoglycan built up by repeating units of *N*-acetyl-D-glucosamine, *N*-acetyl-D-muramic acid and a crosslinking peptide [9–11], and that has no adjuvant property for protein antigens [12].

Wikler [8] has shown that antimicrococcus antibodies are mostly directed against saccharides of the bacterial cell wall and can thus be compared with plant lectins [13]. Their interaction with mammalian cells is

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however more selective than that of lectins as they bind to lymphocytes, to certain tumour cells, but not to erythrocytes [14, 15].

Since the antimicrococcus immunity is characterized by the presence of high titer antibodies of restricted heterogeneity and is subjected to an antigen-dependent clonal expansion, this study was undertaken in mice to investigate if *Micrococcus* prevaccinated mice are able to control the malignant proliferation of leukaemic murine L1210 cells.

MATERIALS AND METHODS

Micrococcus lysodeikticus

A suspension was made of lyophilized *Micrococcus lysodeikticus* (ML) (Worthington Biochem. Corporation) after repeated washings and centrifugations at 3000 ***g*** for 15 min at 4°C, in phosphate buffered saline (0.15 M NaCl–0.01 M potassium phosphate; pH = 7.3) = PBS buffer.

Bacillus Calmette-Guérin

One hundred milligrams containing vials of lyophilized Bacillus Calmette-Guérin (BCG) for scarification were purchased from the Pasteur Institute, Brussels. After recombination with the appropriate diluent and further dilution in Hanks' balanced salt solution, the suspension was kept at 4°C and used within two weeks.

Immunodiffusion

Two dimensional immunodiffusion was performed according to the method of Ouchterlony [16]. Precipitin lines formed at room temperature after 48 hr were stained.

Quantitative agglutination analysis

These were carried out in U shaped wells of Linbro agglutination plates (0.05 ml). The antiserum was mixed with 0.05 ml of phosphate buffered saline and consecutive twofold dilutions were made. After a preincubation period of 30 min at 37°C, 0.05 ml of the micrococcus suspension (2 mg/ml) was added and agglutination was scored after 2 hr at 37°C and expressed in $\log_2$ units.

Electrophoretic procedures

Cellulose acetate electrophoreses were carried out with a Beckman microzone cell model R200. Electrophoresis was carried out in barbital buffer (I = 0.075. pH = 8.6) for 20 min at 250 V at 4°C.

After staining with Ponceau S, the strips were destained with 5% acetic acid and scanned with a Vitatron modular Photometer with scanning accessory.

MICE AND TUMOUR

Mice

Inbred (BALB/c × DBA/2) F_1 animals were supplied by Charles River Breeding Laboratories. Those mice were stored at least for three weeks in an isolation room before we started experimenting. Female CDF_1 mice (female BALB/c × male DBA/2) F_1 animals weighing 19–23 g were used.

Tumour

L1210 lymphoid leukaemia was originally obtained from Mr. I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.).

This tumour was maintained in DBA/2 mice by weekly intraperitoneal (i.p.) inoculation. L1210 cells were aspirated from DBA/2 mice and a suspension was made in Hanks' balanced salt solution: 10^4 cells in 0.1 ml solution were injected intraperitoneally to obtain the ascitic form of leukaemia. Animals were randomized into test groups and control groups.

For each survival experiment, 10–20 mice were used. The mean survival time ± the standard deviation of the treated mice and the control mice as well as the dosage and timing of each experiment are specified in the tables. Survivors on day 90 after tumour grafting were considered as long-term survivors and they are not used in the final evaluation of the mean survival time and the standard deviation (M.S.T. ± S.D.). A 25% increase in the mean survival time of treated mice over controls was considered significant and supported by a single tailed Student Fisher test.

Specific immunotherapy

Tumour cells were harvested from the peritoneal cavity of a DBA/2 mouse bearing a 7-day-old L1210 tumour (10^5 cells grafted on day 0) and grown without antibiotics as suspension cultures in Roswell Park Memorial Institute Medium 1640 (RPMI) supplemented with 10% heat-inactivated foetal calf serum. Tumour cells were routinely passaged every 48 hr as stationary cultures in Falcon 3024 flaks or grown exponentially in spinner cultures 48 hr before experimenting. Cultured cells were spun down and adjusted

to 5.10^7 cells/ml RPMI. After irradiation of 8000 rad (250 kV, 12 mA, 95 rad/min) by X-rays, 10^7 cells were grafted to CDF_1 mice.

RESULTS

1. *Natural antibodies to* Micrococcus lysodeikticus

The mammalian gut and part of the digestive tract are attractive loci for non-pathogenic Gram-positive as Gram-negative bacteria that constitute the "bacterial flora" and live in symbiosis with the mammalian host. Housing of these micro-organisms is immunologically restricted to the gut epithelium.

Since the cell walls of most of these bacteria contain carbohydrates or glycoproteins, we were not surprised to find positive agglutination patterns, presumably due to cross-reactions, with *Micrococcus* cells in 25 out of 25 tested sera of pre-immune rabbits at 4°C and 19 out of 25 pre-immune rabbits at 37°C.

Neither sera taken from 48 preimmune BALB/c mice, nor sera taken from 25 pre-immune C57 black/6j mice agglutinated *Micrococcus* cells at 37°C. However, in 17% of BALB/c mice and 4% of C57 black/6j mice, activity was detected at 4°C. These results strongly suggest that the cross-reacting protein acts as a cryoglobulin with low serum titer and low affinity as physiological temperature.

2. *Origin and evolution of the antimicrococcus immunity*

Previous studies in mice indicated that the immune response is higher when the vaccine is inoculated intraperitoneally [17].

One hundred and three CDF_1 mice were vaccinated with 0.15 mg heat-killed micrococcus cells (60°C for 30 min) once a week over 4 weeks.

They received three injections a week of 0.2 mg during weeks 10–14 and of 0.4 mg during weeks 24–29 and 31–36. Blood samples were taken from the retro-orbital plexus at different stages of the immunization period and their sera were subjected to cellulose acetate electrophoresis to check the presence of immunoglobulins of clonal dominance. The presence of antimicrococcus antibodies in the serum was detected by precipitation with lysozyme solubilized cell walls, in double diffusion (immunodiffusion). By quantitative agglutination with 2 mg/ml *Micrococcus* suspension, the serum antimicrococcus antibody titer of every bleeding was determined. This immunization schedule resulted in the production of antibodies of restricted heterogeneity and clonal dominance in 5% of the mice from week 25, 26% on week 30 and 33% on week 37, as seen in Fig. 1. As seen in Fig. 2, the mouse population first responded to the antigen with antibody titers resembling a binomial distribution (weeks 1–23) that shifts to an asymmetric curve on weeks 30–37. Antimicrococcus antibody titers of homogeneous responders were considerably higher (mean $\log_2$aggl. *Micrococcus* = 6.0) than non-homogeneous responder mice (mean $\log_2$aggl. *Micrococcus* = 2.0) on week 37.

3. In vitro *interaction of mouse antimicrococcus sera with L1210 or Ehrlich carcinoma cells*

Using a Leitz Orthoplan microscope, we found that fluorescein conjugated rabbit anti-micrococcus globulins bind to Ehrlich ascites cells [14] and that fixed (0.3% formaldehyde) carcinoma cells can be agglutinated by rabbit antimicrococcus sera. Controls with pre-immune immunoglobulins or sera were negative in both experiments.

Mice that were injected i.p. with L1210 cells preincubated with antimicrococcus gammaglobulins displayed an increase in mean life span, as compared to control mice that received the same dose of tumour cells incubated with the pre-immune gammaglobulins of the same rabbit [17].

Using a panel of mouse antimicrococcus sera of both homogeneous and non-homogeneous responders, we observed that immune sera of syngeneic mice, unlike pre-immune control sera or bacterium, agglutinate L1210 and Ehrlich carcinoma cells *in vitro*. As outlined in Table 1, agglutinations were performed according to the described quantitative agglutination procedure, using 10^7 cultured L1210 cells per ml and 5.10^6 Ehrlich carcinoma cells per milliliter as target suspensions.

4. *Tumour immunoprophylaxis exerted by the antimicrococcus immunity*

Part of the Micrococcus vaccinated CDF_1 mice were subdivided according to the presence in the serum of antibodies of clonal dominance into homogeneous and non-homogeneous responders on week 39. Both groups and a control group of unvaccinated mice were tumour challenged i.p. with 10^5

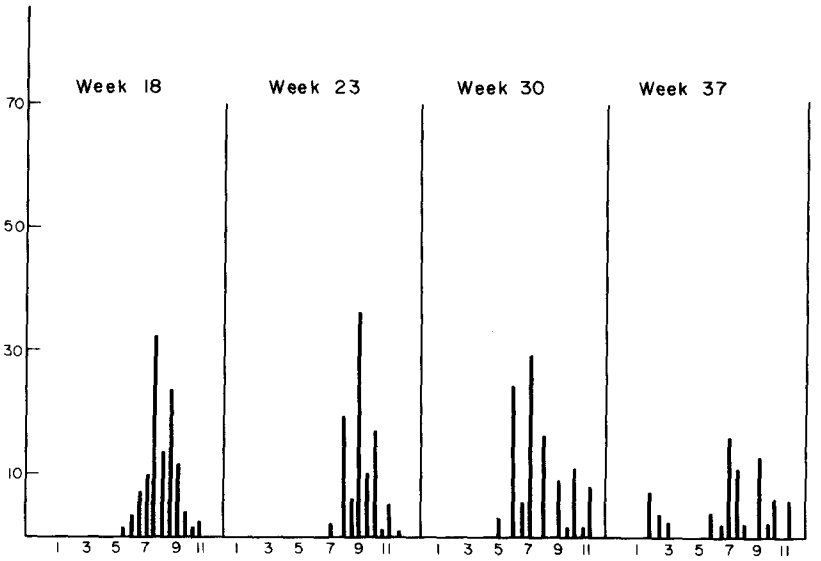

Fig. 2. Evolution of the antimicrococcus antibody response of CDF_1 mice, hyperimmunized with Micrococcus lysodeikticus.
Abscissa: log_2 Micrococcus *agglutination titer.*
Ordinate: Percentage of the mouse population.

Table 1. Comparison of the activity of mouse antimicrococcus sera on Micrococcus and neoplastic murine cells

Log_2 agglutination *Micrococcus*	Log_2 agglutination L1210	Character of the serum	Log_2 agglutination *Micrococcus*	Log_2 agglutination Ehrlich carcinoma	Character of the serum
7.5	1.0	antimicrococcus	9	3.0	antimicrococcus
8.0	1.0	antimicrococcus	9	3.0	antimicrococcus
9.0	1.0	antimicrococcus	8	4.5	antimicrococcus
11.0	1.0	antimicrococcus	9	2.5	antimicrococcus
8.0	0.0	antimicrococcus	9	3.0	antimicrococcus
8.0	0.0	antimicrococcus	9	2.0	antimicrococcus
4.0	0.5	antimicrococcus	9	4.5	antimicrococcus
5.0	0.0	antimicrococcus	8	3.5	antimicrococcus
10.0	3.5	antimicrococcus	8	5.0	antimicrococcus
9.5	>7	antimicrococcus	8	0.0	antimicrococcus
8.0	1.0	antimicrococcus	4.5	1.0	antimicrococcus
0.0	0.0	pre-immune	0.0	0.0	pre-immune

Quantitative agglutinations were performed at 37°C after a preincubation of 30 min.
Quantitative agglutinations were performed with cultured L1210 cells (10^7/ml) or Ehrlich carcinoma cells (5.10^6 cells/ml) at 37°C, as for the *Micrococcus* suspension.

in vivo grown L1210 cells. Another part of the vaccinated population and a control group received a graft of 5.10^3 leukaemic cells i.p. Vaccinated mice did not show any anti-tumour immunoprotection: for all graft dosages, the mean survival time of vaccinated mice is comparable to that of unvaccinated mice.

Since one could hypothesize that the anti-tumour subpopulation of antimicrococcus antibody (whose affinity for tumour cell epitopes is demonstrated by the agglutination test) is transiently present during the immunization period, a new experiment was devised.

Intact CDF_1 mice received i.p. three 0.1 mg injections a week on weeks 1–4, 0.2 mg on weeks 6–9, 0.3 mg on weeks 11–14, 0.4 mg on weeks 16–19 and finally 0.5 mg on weeks 20–24. On weeks 5, 10, 15, 20 and 25, a part of the vaccinated mice was withdrawn from the population, bled in the retro-orbital plexus and tested one week later for immunoprotection against 10^4 L1210 cells implanted intraperitoneally.

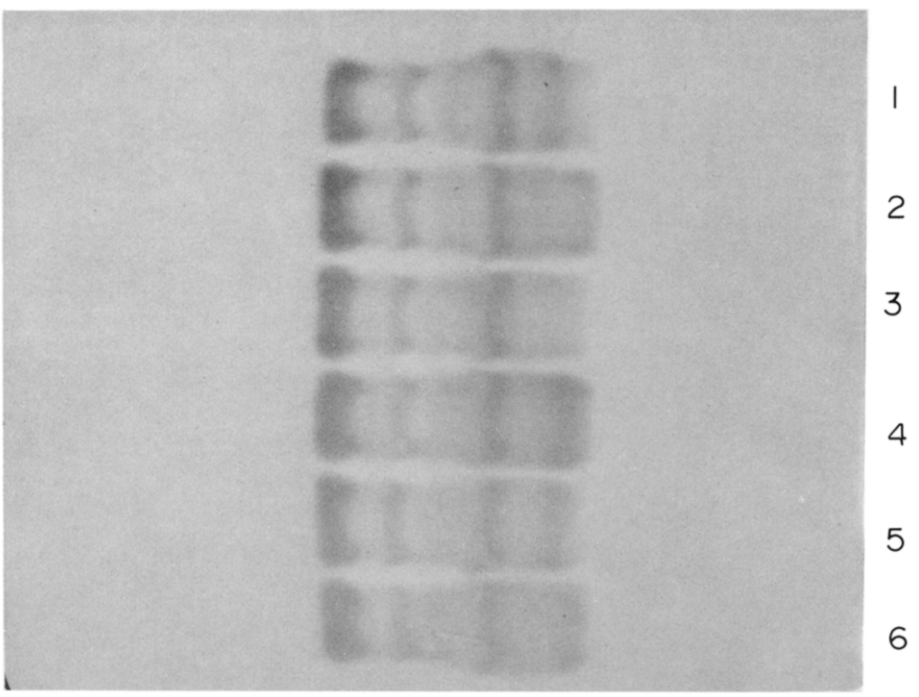

Fig. 1. *Microzone electrophoretic patterns of Micrococcus vaccinated* CDF_1 *mice. Cellulose acetate electrophoreses were carried out with a Beckman microzone cell model R*200. *Electrophoresis was carried out in barbital buffer (Ionic strength* $=0.075$, pH $=8.6$) *for* 20 min *at* 250 V. *The anode is to the left. Notice the presence of immunodominant (clonal dominant or homogeneous) antibodies in the immunoglobulin γ regions of sera* 1, 2, 4, 5 *and* 6 *as compared to pre-immune mouse serum* [3].

Although antimicrococcus antibodies were present from the fifth week in all tested mice, vaccinated mice were never offered a demonstrable anti-leukaemia immunoprotection.

After a rest period of 6 months, we have tested again the immunoprotective capacity of mice vaccinated with micrococcus for 36 weeks. During this period, the humoral antibacterial immune response had slowly decreased, as indicated by comparison of the agglutination patterns obtained from monthly bleedings in the retro-orbital plexus.

was continued by 1 mg injections on days 4, 8 and 12.

Prevaccinated mice that were boosted with micrococcus cells were considerably protected against tumour grafting. The mean life span was significantly ($P<0.001$) increased (M.S.T. = 21.67 days ± 0.58) over the control mice (M.S.T. = 11.3 days ± 0.60) and 57% of long-term survivors were recorded. *Micrococcus* injected on days 4, 8 and 12 in L1210 bearing and unvaccinated mice was unable to induce longterm survivors; yet, it induced an increase in mean survival time (M.S.T.

Table 2. Comparison of anti-L1210 immune protection of prevaccinated and unvaccinated CDF_1 mice treated with Micrococcus *or specific immunotherapy*

Schedule				Mice survival		
ML prevaccination	Treatment with ML	Treatment with B.C.G.	Irradiated cultured cells	M.S.T. ± S.D. (days)	T/C%	% of mice cured on day 90
+	+	−	−	21.67 ± 0.58	192*	57
+	+	−	+	11.33 ± 1.00	100	0
+	−	+	+	12.86 ± 0.90	114	0
+	−	−	+	12.13 ± 1.25	107	0
−	+	−	−	19.44 ± 1.24	172*	0
−	+	−	+	12.86 ± 2.69	114	0
−	−	+	+	13.13 ± 5.11	116	0
−	−	−	+	11.57 ± 2.15	102	0
− (control mice)	−	−	−	11.30 ± 0.60	100	0

CDF_1 mice were vaccinated with *Micrococcus lysodeikticus* during 36 weeks. After a rest period of six months, randomized mice were tumour challenged i.p. with 10^4 leukaemic L1210 cells. Some mice received 1 mg micrococcus injections on days 4, 8 and 12 after tumour grafting, others were treated by a single injection of 10^7 irradiated cultured L1210 cells, alone or in combination with 1 mg BCG or *Micrococcus*. 24 hr after tumour grafting, followed by 1 mg injections on days 4, 8 and 12. Mean survival time (M.S.T.) and the standard deviation (S.D.) of treated mice (T) over control mice (C) are listed and their ratio expressed in percentage (T/C%)
*Significance at $P<0.001$ level.

Micrococcus vaccinated and control mice received 10^4 cells i.p. on day 0. The protective effects of the vaccination with *Micrococcus*, eventually followed by booster injections after tumour challenge, of injection of irradiated cultured L1210 cells or of Bacillus Calmette-Guérin (B.C.G.), were investigated, as illustrated in Table 2.

Part of the vaccinated or control mice received i.p. 1 mg injections of *Micrococcus* on days 4, 8 and 12. Twenty-four hours after tumour challenge, part of vaccinated or control mice received i.p. 10^7 irradiated cultured L1210 cells, alone or mixed with 1 mg of Bacillus Calmette-Guérin (B.C.G.) or 1 mg of *Micrococcus* and the treatment

~19.44 days ± 1.24) as compared to control mice (M.S.T. ~11.3 days ± 0.60) ($P<0.001$). However, all other schedules were unsuccessful, as illustrated in Table 2.

DISCUSSION

In this paper we have studied the immunogenicity of the *Micrococcus*–antimicrococcus system in CDF_1 mice. An immunization schedule that leads to the production of antibodies of restricted electrophoretic heterogeneity and clonal dominance in those mice is described.

In contrast with rabbits where similar mg/kg dosages lead to the presence of homo-

geneous antibodies in the serum after eight weeks of active vaccination, we found that, in mice, immunoglobulins of clonal dominance appear no sooner than the 25th week.

Although Krause *et al.* [18] have demonstrated that the intravenous route is superior to other administration routes in the rabbit, mice were vaccinated intraperitoneally but we do not exclude that other routes may favourably influence the production of those particular immunoglobulins. It is worthy of note that the micrococcus vaccinated population responded to the antigen with antibody titers resembling a symmetric binomial curve until the 30th week. Thereafter, simultaneously with the appearance of antibodies of clonal dominance in the serum of some mice, the distribution curve seemed to be split into two areas: non-homogeneous responders tended to adhere to the low titer responder area, whereas homogeneous responders showed the highest antibody titers. Since L1210 cell membranes are coated with immunoglobulins *in vivo* [19], we decided to use "nude" tumour cells obtained from cell line cultures for the specific immunotherapy and *in vitro* tests. Unlike pre-immune sera, antimicrococcus sera were found to agglutinate non-fixed syngeneic leukaemic L1210 cells as Ehrlich carcinoma cells at physiological temperature.

Therefore we wanted to investigate if a similar binding on tumour cell epitopes due to a cross-reactive immunity might occur *in vivo* and consequently, if micrococcus vaccinated mice should be immunologically resistant to tumour grafting. In our experiments, neither mice producing antimicrococcus antibodies of restricted heterogeneity, nor non-homogeneous responding mice were immunologically protected against 10^5 or 5.10^3 L1210 leukaemic cells.

To exclude the possibility that the antitumor subpopulation of antimicrococcus is transiently present during the immunization period, we vaccinated a new population of intact mice and tested the antitumour immune resistance of those mice at different stages of the immunization period. Again, no anti-L1210 immunoprotection could be demonstrated in those vaccinated mice.

However, since we earlier demonstrated [17] that incubation of L1210 cells with rabbit antimicrococcus gammaglobulins and grafting those cells to intact mice led to the rejection of those incubated cells, unlike L1210 cells incubated with pre-immune globulins of the same rabbit, we hypothesize that the presence of the antimicrococcus antibody *per se* is insufficient to afford an effective immunoprotection. It is possible that the challenged mouse makes an immune response to the rabbit antibody on the surface of L1210 cells. This response could act as a trigger for the immunologic effector mechanisms. Such a trigger can be provided by the complement activating bacteria—*Micrococcus* [20] or Bacillus Calmette–Guérin [21]—and may be also offered by the structure of the Fc part of a xenogeneic antibody (rabbit). Indeed, evidence from multiple sources has been given that clinical as well as murine tumour (including L1210 [22] and Ehrlich carcinoma [23]) growth is accompanied by an impaired T cell function [24] and that mean survival time of skin grafts is considerably prolonged in tumour bearing animals [25]. Cell types known to function in antibody-dependent cellular cytotoxicity include Fc receptor bearing macrophages as well as B and T lymphocytes [26] and many of them, after activation, display membrane receptors for complement [27, 28].

Compatible with our assumption is the observation that booster doses of 1 mg *Micrococcus* to Micrococcus prevaccinated mice on days 4, 8 and 12 are able to induce 57% of long-term survivors cured on day 90 after grafting 10^4 L1210 cells intraperitoneally and to increase significantly ($P<0.001$) mean life span of vaccinated mice over control mice. However, mean life span of non-prevaccinated and Micrococcus treated mice was considerably increased ($P<0.001$) over control mice, but no long-term survivors were recorded. Neither prevaccinated mice given irradiated cultured L1210 cells alone or in combination with B.C.G. or M.L., nor unvaccinated mice given the same treatment were immunoprotected against tumour grafting.

It seems logical to assume that irradiated tumour cells (10^7) injected 24 hr after tumour grafting may compete with tumour grafted cells (10^4) for the antimicrococcus anti-tumour subpopulation *in vivo*, so that the immunoprotective effect is abolished.

It is important to mention that, in contrast to the experiments presented by Mathé [29], lyophilized B.C.G. vials were used in order to compare the activity of the bacterial with the activity generated by heat-killed *Micrococcus lysodeikticus* and furthermore, that mice were treated i.p.

In general, we conclude that the *Micrococcus*–antimicrococcus immunobacterial system is a promising anti-tumour system that, if adequately monitored, may afford a considerable immunoprotection against murine leukaemia tumour growth.

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Daunomycin–Immunoglobulin Conjugates, Uptake and Activity *In Vitro**

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Abstract—*Daunomycin, an anti-tumor drug, the activity of which is known to be exerted mostly in the nucleus, was covalently attached to anti-tumor antibodies or to normal immunoglobulin. The attachment was accomplished either by direct linking or via a dextran bridge. All the conjugates retained a significant amount of their original drug activity. The capacity of these macromolecular conjugates to penetrate the malignant cell and the nucleus was examined. The compounds were found to be able to penetrate the cell membrane in the conjugated form. Daunomycin linked to anti-tumor antibodies entered the cell at a higher rate than daunomycin attached to normal Ig and daunomycin–dextran. In normal cells, no difference in uptake was observed between drug-immunoglobulin or drug-anti-tumor-antibody conjugates. Uptake studies with daunomycin conjugates, radioactively labeled by different methods, suggest that the drug is released from the complex after penetration into the cell but probably enters the nucleus as the free drug.*

INTRODUCTION

THE USE of antibodies [1–4] or other specific [5] and non-specific [3, 5, 6–10] macromolecules as carriers of anti-tumor drugs has been investigated by various groups throughout the last few years. The mode of action of these drugs requires their penetration into the cell and mainly into the nucleus, where they both intercalate and adlineate with nuclear DNA [11, 12]. We have used the anthracyclines daunomycin and adriamycin, linked to anti-tumor immunoglobulins or antibodies [13–15] as specific carriers to target cells, in an attempt to minimize the detrimental effect of these toxic drugs on normal cells. The covalently linked drug-antibody conjugates were shown to retain an appreciable amount of drug activity *in vitro*, and, as found recently, also *in vivo*.

We have since synthesized conjugates in which the linking of the anthracyclines to the antibody was not made directly but through a dextran bridge and thus obtained a high conjugation of the drug to the antibody.

We have attempted to clarify the mechanism of action of the conjugates of the drug with antibody or with immunoglobulin by testing the ability of these complexes to enter the cells. Previously it was shown that antibodies directed against the major histocompatibility locus are able to enter the cell and bind to the nucleus [16]. In this study, the uptake of radioactively labeled derivatives of the conjugates by tumor and normal cells and their nuclei was explored in correlation with their activity *in vitro*.

MATERIALS AND METHODS

Daunomycin–HCl (lot 33) was a gift from Farmitalia (Milano, Italy). Tritiated daunomycin (specific activity 14 Ci/mmole) was a gift from New England Nuclear (Mass., U.S.A.). Sodium boro[^{3}H]hydride (specific activity 293 mCi/mmole), and [5-^{3}H]uridine (specific activity 25 Ci/mmole) were purchased from the Radiochemical Centre (Amersham, England). [6-^{3}H]Thymidine (specific activity 4.5 Ci/mmole) was obtained

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Abbreviations used: PBS, 0.15 M sodium chloride–0.01 M sodium phosphate, pH 7.2; Dau, daunomycin; Dex, dextran; NP40, Nonidet P40; Ig, immunoglobulin; TLC, thin layer chromatography.

from Nuclear Research Center, Dimona, Israel). Sodium borohydride was purchased from Merck (Schuchardt, Germany). Sodium periodate and mannitol were obtained from British Drug House (Poole, Englend). Biogel P-60 was purchased from Bio-Rad (Calif, U.S.A.).

Antibodies were prepared in goats against a soluble papain digest of a tumor cell membrane (Yac lymphoma carried out as a line in A/J mice) [17]. The antibodies were purified by the use of glutaraldehyde-fixed Yac Cells (10^7 cells/ml with 0.1% glutaraldehyde for 90 min at room temperature) as an immunoadsorbent and followed by elution with 2 M NaI (Merck, Darmstadt, Germany), by batch elution twice for 1 hr at 37°C. The normal goat immunoglobulins were precipitated from goat sera by 33% saturated ammonium sulphate.

Preparation of daunomycin conjugates

The binding of daunomycin to antibodies or to normal Ig was performed as previously reported [13], by oxidation of the sugar moiety of the daunomycin with periodate, linking it to the amino groups of the protein, followed by reduction of the Schiff bases with sodium borohydride. The only difference was that in the present study mannitol was added to the pure daunomycin in a ratio of 5/1. The oxidation of daunomycin in the absence of mannitol, even when the concentration of sodium borohydride was decreased from 0.1 to 0.02 M resulted in loss of solubility and lower yields.

The binding of daunomycin to dextran was performed by the use of periodate-oxidized dextrans [18]. The Schiff bases formed between the aldehyde groups on the oxidized dextran and the amino group on the sugar moiety of the drug after an overnight incubation at room temperature, were quantitatively reduced by sodium borohydride for stabilization of the binding.

Daunomycin–dextran-antibody or IgG

Oxidized dextran to which daunomycin had been linked (ratio of daunomycin to dextran 1:2) was incubated overnight with the immunoglobulin fraction or purified antibody preparation (Ig to dextran ratio 1:1) at 4°C and the resulting complex was reduced by $NaBH_4$ as above. Dextran-bound drug or Ab-dextran bound drug were separated from free drug by gel filtration on Biogel P-60. The conjugates were characterized as follows:

The daunomycin was determined by the absorbance at 495 nm, the antibody or Ig by absorbance at 280 nm (the absorbance of the drug at 280 nm was corrected for) and the dextran was quantitated by weight.

Each preparation was checked by thin layer chromatography for the presence of unbound daunomycin. The various conjugates contained between zero and 3.6% of its daunomycin in an unbound form.

Dialysis of the conjugates against 0.1 M acetate buffer at pH 4.2 resulted in release of 20–30% of the drug, indicating that the reduction was incomplete. However, it was impossible to use more drastic conditions for reduction, since this yields a product with damaged daunomycin as indicated by its decoloration.

The daunomycin content in the various preparations was determined to be as follows: daunomycin–antibody conjugates; 2–3 mole/mole; daunomycin–dextran conjugates; approximately 1 mole/mole (of dextran T-10, mol.wt 10,000); and in the daunomycin–dextran–antibody conjugates between 10 and 30 mole/mole antibody.

Determination of drug activity

Daunomycin activity was assayed by the inhibition of either uridine or thymidine incorporation. The drug or its conjugates were incubated with the tumor cells for a period of 2 hr. Either ^{3}H-uridine or ^{3}H-thymidine were then added for 1 hr at 37°C. The analysis of the incorporated nucleotide was performed as described for ^{3}H-uridine [13]. Overnight incubation (20 hr) of the drug or its conjugates in addition to ^{3}H-thymidine gave similar inhibition results to those obtained for the short incubation period.

^{3}H-labeling of daunomycin

In addition to tritiated daunomycin (New England Nuclear), daunomycin was labeled either by oxidizing its sugar moiety with periodate and then reducing the resulting aldehyde groups by NaB^3H_4, or by direct treatment with NaB^3H_4 without prior oxidation. The labeled preparations were checked by comparison of their chromatographic behaviour in TLC (in chloroform:methanol: water 13:6:1) to that of unmodified daunomycin. The R_F of the oxidized and reduced daunomycin was 0.66, that of the NaB^3H_4 treated material was 0.68 as compared to R_F 0.57 of cold daunomycin. The oxidized and reduced daunomycin lost an appreciable

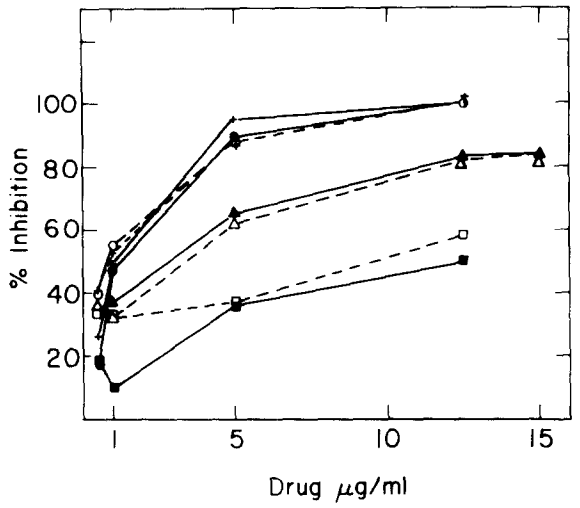

Fig. 1. Daunomycin and daunomycin–dextran activity determined by inhibition of ^{3}H-*uridine (broken line) and* ^{3}H-*thymidine (continuous line) incorporation. (+) daunomycin; (◑) daunomycin treated by* $NaBH_4$ *(◨) daunomycin oxidized by periodate and reduced by* $NaBH_4$*(▲) daunomycin–dextran (dextran* T40, 50% *) periodate oxidized.*

part of its drug activity, but the $NaBH_4$ treated compound was as active as the original daunomycin (Fig. 1).

The stability of the radioactive label was assessed by incubation of the labeled compounds at pH values 4.7 and 9.5, respectively, for 24 hr, after which the solutions were absorbed on a poropak Q column, and both efluent and eluent were monitored for radioactivity. Almost no loss in the radioactivity (~0.3%) was observed in acidic or neutral pH. Under basic conditions (pH 9.5) a loss of about 9% was obtained. These results show that the stability of the label in the daunomycin is quite high and should not interfere with the uptake experiments. This is true both when the label is in the sugar moiety (in the oxidized and reduced preparation) and when it is in the quinone ring or the keto side chain (in the reduced product).

Radioactive labeling of daunomycin–dextran and daunomycin–dextran–antibody conjugates

The conjugates were labeled with tritium by different procedures: (1) Tritiated sodium borohydride reduction of the Schiff bases formed during the binding process. (2) Preparation of the conjugates with tritiated daunomycin (catalytic exchange with ^{3}H gas). (3) Combined tritiated daunomycin and reduction by NaB^3H_4. The stability of the label was determined by dialysis of the compound against PBS for 6 hr and monitoring the radioactivity both inside and outside of the dialysis bag. The loss of radioactivity during the dialysis was approximately 4%. When the labeled conjugate was tested by TLC, the amount of radioactivity retained in the origin was about 70% of the total.

The cellular uptake of daunomycin and daunomycin conjugates

The uptake of daunomycin and its conjugates into cells, and their intracellular distribution, were tested by incubating 5×10^6 tumor or normal cells, with various concentrations of the radioactively labeled compounds, at 37°C and for several periods of time mostly up to 4 hr. At the termination of the incubation period the cells were washed extensively, and the extracellular unbound drug or drug-conjugates were counted. The cells were then incubated for 30 min in 1% Nonidet P40 at 4°C, followed by centrifugation (Sorvall 15,000 rev/min for 10 min). An aliquot (0.1 ml) of the supernatant, which included the fraction rich in cellular membranes and cytoplasmic contents, was counted. The pellets composed of the nuclei rich fraction were dispersed in 0.1–0.2 ml 1N NaOH and transferred into scintillation vials for counting. The scintillation fluid contained a mixture of toluene, tritone X-100 and 0.1 N HCl at a ratio of 6:3:1.

Testing the stability of daunomycin conjugates in the presence of cells

Yac cells (1×10^7) were incubated for various periods with radioactively labeled daunomycin conjugates. Incubation was followed by centrifugation. Aliquots of the supernatants were applied to columns of biogel P-60. The antibody-bound and the unconjugated drug were detected according to their elution position from the columns and quantitated by radioactivity and by optical density at 495 nm. The same tests were performed with the NP-40 supernatants of the postincubated washed tumor cells.

RESULTS

The activity of daunomycin and its various conjugates as measured *in vitro* is shown in Table 1 and Figs. 1 and 3. The activity was determined by inhibition of either ^{3}H-uridine or ^{3}H-thymidine incorporation. When daunomycin was directly linked to anti-Yac antibody or to normal Ig, an appreciable amount of its original activity was retained in the conjugates. Linking of daunomycin

Table 1. In vitro *activity of daunomycin and daunomycin-conjugates*

Dau conc. μg/ml	% inhibition of ^{3}H-uridine incorporation*					
	Dau	Dau–dex	Dau–dex–anti-Yac	Dau–dex–NIg	Dau–anti-Yac	Dau–NIg
0.25	11	0	–	–	–	–
0.50	42	2	8	4	22	39
1.00	60	50	9	17	44	62
5.00	90	80	44	48	86	90
15.00	–	–	63	58	–	–
50.00	–	–	90	92	–	–

*Yac cells (10^6) were incubated for 2hr at 37°C in the presence of daunomycin or daunomycin-conjugates. Incorporation of [^{3}H]-uridine into cellular material precipitable by trichloroacetic acid was measured and expressed as per cent inhibition (100% of control culture containing no drug).

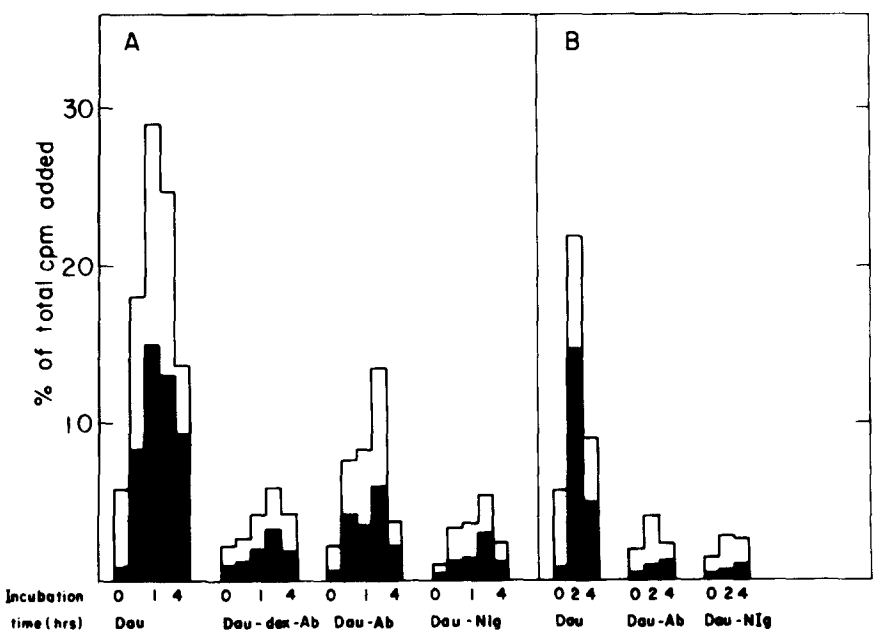

Fig. 2. *The* in vitro *uptake of daunomycin and its conjugates by Yac cells (A) and normal rat lymphocytes (B), measured as a function of time. Measurements of uptake were made at zero time and after* 0.5, 1, 2, 3 *and* 4 hr *following the addition of drug. The total drug accumulation in the cells is denoted by the open bars, whereas the nuclear incorporation by the shaded areas.*

to dextran resulted in a reduction of drug activity and when the dextran served as a bridge between the drug and anti-Yac a further decrease in the capacity to inhibit uridine and thymidine incorporation was observed. Daunomycin, in which the amino sugar was periodate-oxidized and reduced, also showed a decrease in activity, whereas the mere treatment by sodium borohydride had no apparent effect on the *in vitro* activity of the compound.

The uptake of daunomycin and its conjugates by Yac or normal cells (rat splenocytes) is depicted in Fig. 2A and B. It is expressed as % uptake of the total radioactivity added to a fixed number of cells. The distribution of daunomycin or daunomycin-conjugates in the membranes or the cytoplasm and its possible attachment to the nucleus was measured as a function of time of incubation. As can be seen, the free drug controls; daunomycin, oxidized and NaB^3H_4 reduced, or NaB^3H_4 treated, penetrated the cells very rapidly. Since the uptake assay was performed by centrifugation and washing at 0°C immediately after the addition of the drug, high intracellular levels of the free drug were observed at the zero point. Optimal levels of uptake was achieved at a drug concentration of 15–30 μg/ml, with a slight drop at 60 μg/ml. Uptake of the free drug reached a peak at 1 hr incubation, and declined after 3 hr. The accumulation of the drug in or on the

nuclei (shaded areas in the chart) paralled its uptake by the intact cell and amounted roughly to two thirds of the total cellular content.

^{3}H-daunomycin, prepared by catalytic exchange, behaved somewhat differently; i.e. most of the drug added (90% or more) penetrated the nucleus rapidly. In addition, no reduction in its uptake was noted after 3 hr and no decline in its uptake was observed at a concentration of 60 μg/ml.

Daunomycin–antibody conjugates labeled by NaB^3H_4 reduction, whether directly bound or via a dextran bridge, penetrated into the Yac cells and attached to their nuclei, although at a much lower rate than that of the free drug (Fig. 2A). As depicted by the results, daunomycin bound directly to antibodies entered the cells at higher levels than the drug bond, through dextran, to normal Ig. The optimal concentrations were similar to those obtained for free drug without an observed dependence on the amount of antibody in the preparation (the range of concentration tested with 1–3 mg/ml). Drug bound to antibody via the dextran bridge was taken up somewhat more readily at higher concentrations (60–80 μg/ml). The uptake of the bound substances proceeded at a slower rate than that of free drug, peaked at 2–3 hr and slightly decreased after 4 hr. Except for the decline after 4 hr, this corresponds well with the activities of the bound and free drug (Fig. 3). In assays of drug activity maximal levels are reached by the free drug after approximately 90 min, while longer incubation periods (up to 4 hr) are needed for daunomycin conjugate with either dextran or with antibodies via dextran for reaching their maximal potency. As is also depicted in Fig. 2, the accumulation of drug on the nuclei paralleled uptake by whole cells, comprising half to two thirds its value.

In normal non-related cells (rat splenocytes) the uptake of free drug was slightly lower than that by the tumor cells. The daunomycin conjugates with either anti-Yac antibodies or normal Ig (linked directly), were taken up at low rates (Fig. 2B) and did not differ from each other. This was expected, since these cells do not bind goat anti-Yac.

Daunomycin–dextran and daunomycin–dextran–antibody conjugates were tested for their stability in their intracellular and extracellular form. As can be seen from Table 2 both compounds preserve the conjugated form in the presence of cells up to 24 hr. After cell penetration an appreciable amount of free drug remains bound to the antibody dextran (70–75%) and to the dextran alone (43–44%) in the detergent supernatant. In each case the antibody in the conjugate adds to the stability of the complex.

The uptake of conjugates labeled at different positions in the complex is depicted in Fig. 4. In conjugates, whereby only the drug is radioactive, the label accumulates mostly (90%) in the nucleus. On the other hand, in conjugates labeled at the attachment

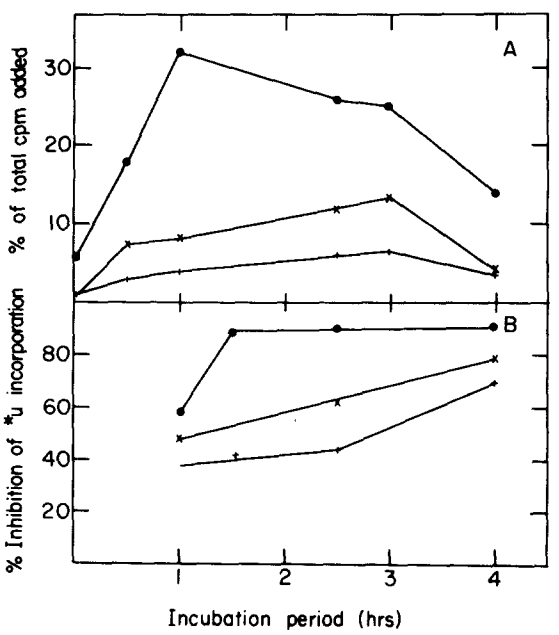

Fig. 3. The relationship between daunomycin or its conjugates uptake (A) and activity (B) as a function of the incubation period with Yac cells. (●) daunomycin; (×) daunomycin-anti-Yac; (+) daunomycin–dextran–anti-Yac. Concentration of daunomycin free or in the conjugates is 5 μg/ml.

Table 2. Stability of daunomycin conjugates in the presence of Yac cells and after their penetration into the cells

	Conjugated drug, % of total*			
	Cell supernatant†		NP_{40} supernatant‡	
	Incubation (hr)		Incubation (hr)	
	3	24	3	24
^{3}H-dau–dex	65	52	43	49
^{3}H-dau–dex-Ab	95	89	70	75

*The results reported are an average of two experiments.

†Yac tumor cells were incubated for 3 and 24 hr with the conjugates and the supernatants were applied to biogel P-60 columns and analyzed.

‡The above cells were extensively washed and incubated for 30 min in 0.5 ml 1% NP_{40}. Aliquots of these supernatants were applied to biogel P-60, columns and analyzed.

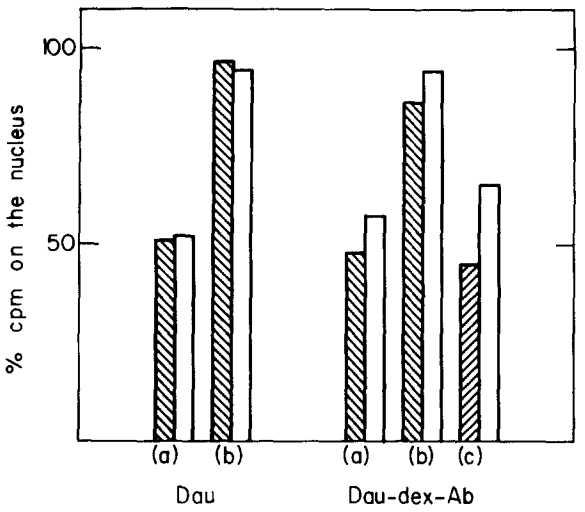

Fig. 4. Daunomycin and daunomycin-conjugates in or on the nucleus after cell penetration. The percent of cpm on the nucleus out of the total penetration cpm was measured with differently labeled compounds. Dau: (a) NaB^3H_4 treated (b) Tritiated. Daunomycin–dextran–anti-Yac (Ab): Reduced by NaB^3H_4. (b) Conjugated with tritiated daunomycin. (c) Both conjugated with tritiated daunomycin and reduced by NaB^3H_4. Incubation for 1 hr; *shaded area, for* 3 hr; *open area.*

point between the drug and the macromolecules total radioactivity is divided between the cytoplasmic contents and the nucleus (only about half to two thirds in the latter). Similarly, when tritiated daunomycin was bound to dextran or to dextran-antibody, and the reduction was performed with tritiated sodium borohydride, radioactivity was similarly divided between cytoplasmic and nuclear fractions.

DISCUSSION

Daunomycin is known to react with polynucleotide compounds. The intercalation and adlineation of daunomycin and similar derivatives to DNA was established by various physico-chemical methods. Thus, the cellular activity of the drug is assumed to be mainly nuclear in nature, causing the unavailability of template DNA both to DNA and RNA polymerases [11, 12]. Whether this describes the full scope of the drug activity is not entirely clear. Various data and experimental observations seem to indicate that a wider spectrum of activities could be attributed to daunomycin, including undesirable effects such as cardiac toxicity and carcinogenesis [19], in addition to the observed beneficial effects, i.e. cytotoxicity to tumors. Antimitotic activity and cell destruction was observed with doses ineffective as inhibitors of macromolecular synthesis. In uptake studies most of the drug was accumulated in the nuclei and only a small amount in the mitochondria and microsomal fractions. However, chemical modifications, such as those shown by Israel *et al.* [20], showed that very active derivatives were mostly located in the cytoplasm [21] and not in the nucleus.

In the experiments carried out in this study tritium-labeled daunomycin and its conjugates were used. The stability of the label in all the compounds was checked in order to ensure that the extent of tritium exchange during the 4 hr required for uptake experiments, will not interfere with the experiments or affect their interpretation. The conjugates were found to be stable in the presence of cells during the experiment (Table 2).

The binding of daunomycin to various macromolecules affects its capacity to be taken up by the tumor cells and their nuclei (Fig. 2). Direct daunomycin–antibody conjugates were taken up more readily than those prepared via a dextran bridge. Both of the latter were taken up better than their controls, daunomycin–normal Ig and daunomycin–dextran (daunomycin–dextran was completely excluded from the cells in some preparations). In preliminary experiments with double labeled anti-Yac-daunomycin, where the anti-Yac was labeled by ^{125}I and the drug by ^3H-, both labels were found to be located on the nucleus.

The extent of label found on the nuclei amounted to 50–60% of the total intracellular drug content. The per cent of drug-conjugates taken up by the cells did not change appreciably with increasing concentrations. The uptake was more dependent on concentration with free tritiated daunomycin, similarly to observations of uptake with Hela cells [22]. Free drug uptake is maximal after 1 hr incubation and declines gradually after 3 hr. This is in agreement with results observed with L1210 and 3T12 cell lines [11, 13].

Daunomycin bound to anti-tumor antibodies was taken up more effectively by respective tumor cells than by normal rat splenocytes. With the normal cells no difference was observed between daunomycin–anti-tumor antibodies or daunomycin–immunoglobulin. These findings emphasize the contribution of the surface contact to the uptake by the cells.

No close correlation is observed between the uptake capacity and the inhibitory activity (Fig. 3). All conjugate types retained

a significant amount of the original drug activity. The daunomycin–dextran conjugate was a better inhibitor of nucleotide incorporation than daunomycin–dextran–antibody conjugates (Table 1). All conjugates prepared were at least as active as the free drug in the suppression of tumour development *in vivo*, and daunomycin–dextran and daunomycin–dextran–antibody were in many instances even better [24]. Thus, the cytotoxic activity of the conjugates is not directly related to the penetration of the conjugates into the cells.

The mechanism by which the covalently bound daunomycin conjugates exert their intracellular activity is probably similar to that believed to operate for the non-covalent daunomycin–DNA complexes. The first step in this process is pinocytosis, for which affinity of the complex to the cell surface plays an important role and facilitates the entrapment by the cells. Hence, daunomycin–dextran or daunomycin–dextran–Ig which have no surface affinity to either tumor or normal cells are taken up at lower levels than daunomycin linked to specific antibodies.

Daunomycin directly linked to antibody could be envisaged to enter the nucleus in the conjugate form, or, alternatively, it might be released from the carrier prior to its penetration into the nucleus. The different labeling methods used in our study suggest that the latter process takes place. When most of the radioactive label was in the dextran bridges between daunomycin and antibody, incorporation studies showed that about half of the incorporated radioactivity was found in the cytoplasm and only half to two thirds attached to the nucleus. On the other hand, when tritiated daunomycin was bound to the conjugate so that all the radioactivity was located on the drug, most of the label was found in the nucleus after 3 hr of incubation. When the two labeling methods were combined, uptake was again divided between cytoplasmic contents and nucleus (Fig. 4). Furthermore, linking daunomycin to antibodies by stable peptide bonds, such as direct coupling with carbodiimide between antibody and drug [13] or between an *N*-succinyl derivative of daunomycin coupled to the antibody via *N*-hydroxysuccinimide ester, no daunomycin activity could be demonstrated. The succinyl-daunomycin itself was as active as the original drug (unpublished results). Therefore, it seems that daunomycin, in order to convey its inhibitory activity, is released from its conjugates by acid or enzymatic hydrolysis. Further studies to elucidate the mechanism of action of daunomycin conjugates are in progress.

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Endocrine Status in Normal British, Japanese and Hawaiian-Japanese Women

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Abstract—*A pilot study has been carried out on the endocrine status of Hawaiian-Japanese, Japanese and British women. There were no striking differences between the three racial groups when ovarian and pituitary hormone levels were compared. British women had significantly higher plasma and urinary adrenal androgen levels and excreted more urinary corticosteroids than Japanese women: Hawaiian-Japanese women tended to have intermediate levels of these hormones. This effect of environment on endocrine status and its relationship to the risk of breast cancer is discussed.*

INTRODUCTION

WE HAVE been unable to find convincing differences between the endocrine status of normal British and Japanese women aged 16–70 yr that would account for the differences in the incidence of breast cancer between the two races [1–3]. The incidence of breast cancer in Hawaiian women of Japanese ancestry is now similar to that of Caucasians [4] as is that of Japanese immigrants to California [5]. If our results on Japanese and British women are correct, it might be expected that there would be no major differences in the endocrine function of women with Japanese ancestry in Hawaii and the pilot study reported in this paper is an examination of this thesis.

MATERIALS AND METHODS

Subjects

The women studied were divided into three menstrual groups according to menstrual status, pre-menopausal women were those aged 35 yr or more who were still menstruating regularly; "menopausal women" were those whose last menstrual period was more than 6 months but not more than $5\frac{1}{2}$ yr before the date of entry into the study, and "post-menopausal women" were those whose last menstrual period was more than $5\frac{1}{2}$ yr before entry into the study. Women taking steroidal contraceptives within a year of entry or with a history of endocrine or cardiovascular disease were excluded. All British and Japanese volunteers were obtained by personal contact and the Hawaiian-Japanese women were the wives of Japan-Hawaii Cancer Study cohort men, whose names were selected at random and asked to volunteer as study subjects.

The number of women, their mean ages, heights and weights are shown in Table 1. It can be seen that within a given menstrual category the mean and standard deviations of the ages are similar. The Hawaiian-Japanese on average are intermediate in height and weight to British and Japanese women. Both the Hawaiian-Japanese and Japanese women are significantly shorter than British women, although only the Japanese are significantly lighter than British women. There is no significant difference in either height or weight between Japanese and Hawaiian-Japanese women. Although the mean heights and weights for the three racial groups are similar, it could be argued that these figures could hide a disproportionate number of obese or thin women in any one group. Table 2 shows that this is unlikely since the mean body build (expressed as percentage of ideal body weight [6]), is similar in all groups studied.

Collection of blood and urine specimens

In pre-menopausal women the blood and urine was collected between the 16th and

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Table 1. *Age, height and weight of women studied*

Category	Pre-menopausal	Menopausal	Post-menopausal
British *n*	30	20	30
Age	42±4	52±4	59±5
Height (cm)	161±6	160±8	160±5
Weight (kg)	60±9	64±9	63±6
Japanese *n*	28	19	35
Age	43±5	53±4	60±6
Height (cm)	151±5	151±5	148±5
Weight (kg)	53±6	51±8	53±8
Hawaiian-Japanese *n*	13	21	20
Age	45±5	54±2	59±4
Height (cm)	154±3	152±7	150±6
Weight (kg)	55±7	56±11	57±9

All results are expressed as mean±S.D.
n refers to number of subjects.

Table 2. *Body-build characteristics*

Category	Pre-menopausal	Menopausal	Post-menopausal
British	99±13 (30)	105±14 (20)	105±12 (30)
Japanese	104±12 (28)	99±18 (19)	111±19 (35)
Hawaiian-Japanese	102±14 (13)	109±20 (21)	116±23 (20)

Results are expressed as (actual body weight divided by ideal body weight) ×100±S.D. Ideal body weight has been taken as height (cm)−100 (6). Figures in parentheses are the number of women in each group.

24th day of the menstrual cycle. On the first day of study, blood was taken into a heparinized container and plasma separated. In Britain and Japan blood was taken between 11.00 and 17.00 hr and in Hawaii at any time during the morning. Urine was collected for 3 days commencing on the 2nd day of study. After collection the urine and plasma were stored at −20°C. The Japanese and Hawaiian-Japanese specimens, packed in solid CO_2, were air freighted to London and arrived frozen. The samples were then stored at −20°C until analysed. Urine specimens having less than 0.7 g/24 hr of creatinine were discarded as being incomplete collections.

Assays

Plasma oestradiol and oestrone were determined using rabbit uterine cytosol in a modification of a method [7] described by Korenman *et al.* [8] in 1969. Plasma progesterone and urinary pregnanediol were measured using the methods of Swain [9] and Moore and Cooper [10] respectively. Urinary oestrogens were estimated by the method of Salokangas and Bulbrook [11] except that recovery was estimated by using tritium labelled steroids and corrected to 100%.

Plasma LH, FSH, TSH and prolactin were all measured by conventional double-antibody radio-immunoassay. The antiserum to FSH was kindly supplied by Dr. W. R. Butt and that to LH by The National Institutes of Health, Bethesda. Gonadotrophins for iodination were supplied by The Medical Research Council, (FSH, MRC 69/104; LH, MRC 71/53). The concentration of FSH and LH were expressed in terms of the MRC standards 68/39 (32.8 i.u./ampoule) and 68/40 (77 i.u./ampoule), respectively. The reagents and protocol for TSH and prolactin were kindly supplied by the National Pituitary Agency, National Institutes of Health, Bethesda.

Plasma dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) were measured using gas–liquid chromatography

[12]. The concentration of plasma androstenedione was estimated by a radio-immunoassay method outlined previously [13]. The urinary 11-deoxy-17-ketosteroids (11-DKS), androsterone, aetiocholanolone and dehydroepiandrosterone were determined by the method of Bulbrook [14].

RESULTS

The amounts of ovarian and pituitary hormones in the blood of normal British, Japanese and Hawaiian-Japanese women are given in Table 3. Because of the large number of "*t*" tests performed only levels of $P<0.01$ are considered to be significant. The urinary oestrogen and progesterone metabolites are also shown. All results are expressed in geometric means (with the exception of the oestriol quotient [15]). The salient points emerging from the results in the table are that there are no differences between plasma and urinary oestrogens in pre-menopausal women in the three racial groups. The menopausal Hawaiians have a significantly higher plasma oestrone and lower progesterone than British or Japanese women and the urinary excretion of oestrone follows the same patterns as the plasma precursor. In post-menopausal Hawaiians the plasma oestradiol levels were significantly lower than in the other two groups. Oestriol quotients are not different.

The only differences found in the geometric mean levels of plasma LH was in the pre-menopausal group where the Hawaiian women had a significantly higher mean plasma LH level than the British women.

The geometric mean plasma FSH levels were similar for all racial groups except in the post-menopausal group where Japanese women had significantly lower levels than Hawaiian women.

Prolactin levels were similar in all three racial and menopausal status groups.

As far as TSH is concerned, the menopausal Hawaiian women show a significant increase in concentration compared to similar Japanese women.

Androgens

The geometric mean levels of plasma and urinary androgens are shown in Table 4.

Since the androgens are more highly age-related than menopausal-related, regressions of androgen levels on age were calculated.

The logarithms of the DS, AS, androstenedione and 11-DKS values were highly significantly correlated with age in all three racial groups, with the sole exception that in Hawaiian-Japanese women androstenedione did not correlate with age, almost certainly because of the small number of subjects.

When the age-adjusted means calculated from the best-fit line are compared, both Hawaiian-Japanese and British women have significantly higher levels of DS than Japanese women ($F=20.5$; $P<0.001$ and $F=13.0$; $P<0.001$ respectively). Conversely, the Hawaiians resemble the native Japanese in that their androstenedione levels are significantly lower than those in British women (British vs Japanese $F=24.0$, $P<0.001$; British vs Hawaiians, $F=14.6$, $P<0.001$).

The amount of 11-DKS excreted by the three groups is similar to the pattern for androstenedione in that the Japanese and Hawaiians excrete significantly less of these compounds than the British ($F=22.5$, $P<0.001$; $F=7.4$, $P<0.01$).

This trend was observed for both urinary androsterone and aetiocholanolone, although the difference in androsterone excretion between Hawaiians and British women was not significant.

Ratio of androsterone (5α) *and aetiocholanolone* (5β) *excretion*

The mean arithmetic ratio of androsterone to aetiocholanolone (5α/5β) is significantly lower in British women compared to Japanese women in all the age groups studied ($P<0.01$, <0.01 and <0.001 for pre-menopausal, menopausal and post-menopausal groups, respectively). This ratio for Hawaiian-Japanese women lies intermediate between the British and Japanese groups for all age groups (see Fig. 1) and is not significantly different from either race.

Urinary 17-hydroxycorticosteroids

The amount of 17-hydroxycorticosteroids excreted by British women was greater than that for Japanese in all menstrual groups, although the difference was only statistically significant between the pre-menopausal groups. The Hawaiian-Japanese excreted intermediate levels of 17-hydroxycorticosteroids.

DISCUSSION

In pre-menopausal Hawaiian women, the only difference noted was that the LH

Table 3. Ovarian and pituitary hormones in normal British, Japanese and Hawaiian-Japanese women

		British	*n*	Japanese	*n*	Hawaiian-Japanese	*n*
P–E_1	(a)	1.82±0.19	29	1.68±0.32	14	1.72±0.45	9
(pg/ml)	(b)	1.34±0.23	19	1.28±0.24	9	1.54±0.17	10**††
	(c)	1.20±0.22	30	1.18±0.28	20	1.18±0.07	10
P–E_2	(a)	1.96±0.31	29	1.85±0.41	24	1.77±0.29	9
(pg/ml)	(b)	1.00±0.33	19	0.90±0.32	19	0.85±0.44	10
	(c)	0.85±0.29	30	0.90±0.23	29	0.40±0.23	10**††
U–E_1	(a)	0.91±0.22	30	0.86±0.29	27	0.87±0.24	15
(μg/24 hr)	(b)	0.23±0.11	12	0.32±0.22	16	0.57±0.24	5**
	(c)	0.20±0.16	20	0.26±0.34	19	0.23±0.31	11
U–E_2	(a)	0.48±0.19	30	0.30±0.45	27	0.51±0.15	15
(μg/24 hr)	(b)	$\bar{1}$.78±0.48	12	0.00±0.42	16	0.15±0.51	5
	(c)	$\bar{1}$.85±0.33	20	$\bar{1}$.78±0.54	19	$\bar{1}$.85±0.30	11
U–E_3	(a)	0.95±0.35	30	1.00±0.32	27	0.96±0.28	15
(μg/24 hr)	(b)	0.23±0.33	12	$\bar{1}$.90±0.44	16	0.40±0.48	5
	(c)	0.04±0.42	20	0.08±0.57	19	0.18±0.20	11
EQ	(a)	0.45±0.14	30	0.51±0.11	27	0.46±0.19	15
	(b)	0.44±0.15	13	0.24±0.18	16	0.33±0.11	5
	(c)	0.35±0.17	20	0.33±0.21	19	0.40±0.19	11
P–Prog.	(a)	0.94±0.35	30	0.77±0.43	27	0.86±0.45	16
(ng/ml)	(b)	$\bar{1}$.78±0.18	17	$\bar{1}$.95±0.12	15	0.20±0.48	9**
	(c)	$\bar{1}$.90±0.14	22	$\bar{1}$.85±0.20	25	0.08±0.43	9
U–P″-diol	(a)	0.26±0.34	30	0.08±0.40	25	$\bar{1}$.95±0.44	14
(mg/24 hr)	(b)	$\bar{1}$.30±0.30	12	$\bar{1}$.30±0.18	18	$\bar{1}$.30±0.30	10
	(c)	$\bar{1}$.48±0.12	20	$\bar{1}$.30±0.30	19	$\bar{1}$.30±0.18	10
P–LH	(a)	0.67±0.43	28	0.99±0.38	18	1.06±0.28	13**
(m i.u./ml)	(b)	1.72±0.14	12	1.68±0.09	12	1.78±0.18	13
	(c)	1.68±0.11	14	1.59±0.14	14	1.63±0.14	13
P–FSH	(a)	0.52±0.27	26	0.48±0.40	21	0.49±0.35	16
(m i.u./ml)	(b)	1.52±0.25	18	1.48±0.16	17	1.54±0.22	16
	(c)	1.36±0.16	20	1.24±0.16	19	1.46±0.18	13†††
P–HPR	(a)	1.13±0.23	26	1.22±0.23	25	1.24±0.30	13
(ng/ml)	(b)	0.95±0.36	19	0.91±0.20	17	1.10±0.50	21
	(c)	0.98±0.28	29	0.92±0.34	29	0.89±0.31	20
P–TSH	(a)	0.08±0.26	27	0.04±0.21	25	0.23±0.27	13
(ng/ml)	(b)	0.20±0.41	19	0.00±0.18	17	0.28±0.31	21**††
	(c)	0.15±0.27	29	0.00±0.23	29	0.15±0.32	20

P—plasma; U—urinary metabolites. E_1—oestrone; E_2—oestradiol-17β; E_3—oestriol; EQ—oestriol quotient. Prog—progesterone; P″-diol—pregnanediol; LH—luteinizing hormone; FSH—follicle-stimulating hormone; HPR—prolactin; TSH—thyroid stimulating hormone.

Statistical comparisons
British vs Japanese or Hawaiian-Japanese: ** <0.01; *** <0.001.
Japanese vs Hawaiian vs Hawaiian-Japanese: †† <0.01; ††† <0.001.

All results are given as logarithms to the base 10.

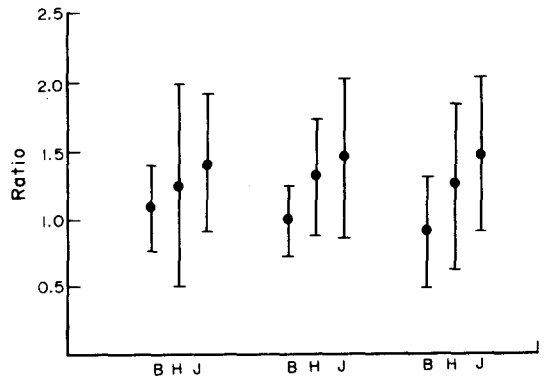

Fig. 1. The ratio of urinary androsterone (5α) to aetiocholanolone (5β) excretion in normal British, Japanese and Hawaiian-Japanese women. The mean±S.D. of the 5α/5β ratio for premenopausal, menopausal and postmenopausal British (B), Hawaiian-Japanese (H) and Japanese (J) normal women are shown from left to right, respectively.

levels were higher than those in British women. No differences were found in other ovarian or pituitary hormone concentrations in British, Japanese and Hawaiian-Japanese women. No pattern emerges that would enable us to relate endocrine status to the three different incidence rates: indeed, if ovarian and pituitary hormone levels were a determinant factor in the incidence of breast cancer, it would be predicted that the future rate in all 3 racial groups would be identical. It might be worth commenting on the fact that there are no differences in prolactin levels in the three groups since so much has been made of the importance of this hormone in carcinogenesis [16–18]. These results are in agreement with literature

Table 4. Adrenal hormones in normal British, Japanese and Hawaiian-Japanese women

		British	*n*	Japanese	*n*	Hawaiian-Japanese	*n*
DS	(a)	1.98±0.20	30	1.80±0.24	27	1.96±0.15	17
(μg/100 ml)	(b)	1.76±0.27	20	1.71±0.28	19	1.84±0.28	18
	(c)	1.74±0.27	30	1.55±0.23	34	1.68±0.23	19
AS	(a)	1.47±0.23	30	1.37±0.21	27	1.50±0.30	17
(μg/100 ml)	(b)	1.30±0.42	20	1.31±0.33	19	1.56±0.30	18
	(c)	1.34±0.33	30	1.26±0.26	34	1.29±0.35	19
Δ4	(a)	2.03±0.11	29	1.97±0.14	27	1.92±0.25	14
(ng/100 ml)	(b)	1.92±0.14	20	1.77±0.17	18	1.81±0.20	12
	(c)	1.94±0.10	30	1.71±0.23	31	1.77±0.28	10
11 DKS	(a)	3.57±0.20	30	3.40±0.22	28	3.39±0.20	17
(μg/24 hr)	(b)	3.34±0.21	18	3.29±0.18	19	3.36±0.20	12
	(c)	3.38±0.26	29	3.13±0.24	34	3.10±0.31	12
A	(a)	3.19±0.22	30	3.06±0.23	28	3.05±0.24	15
(μg/24 hr)	(b)	3.00±0.21	18	3.01±0.20	19	3.11±0.29	7
	(c)	3.00±0.26	29	2.82±0.25	34	2.81±0.36	8
Ae	(a)	3.18±0.18	30	2.95±0.20	28	3.02±0.21	15
(μg/24 hr)	(b)	3.00±0.20	18	2.89±0.21	19	3.02±0.20	7
	(c)	3.08±0.27	29	2.69±0.25	34	2.75±0.35	8
DHA	(a)	2.50±0.69	30	2.40±0.61	28	2.37±0.43	15
(μg/24 hr)	(b)	1.92±0.68	18	2.09±0.66	19	2.33±0.24	7
	(c)	1.88±0.68	29	1.79±0.61	34	2.05±0.71	8
17 OHCS	(a)	0.90±0.13	30	0.50±0.21	25***	0.80±0.12	17†††
(mg/24 hr)	(b)	0.77±0.18	19	0.70±0.14	19	0.81±0.12	13
	(c)	0.81±0.12	30	0.71±0.15	23**	0.69±0.21	12
5α/5β	(a)	1.08±0.32	30	1.40±0.52	28**	1.24±0.74	15
	(b)	1.00±0.27	18	1.43±0.59	19**	1.30±0.44	7
	(c)	0.91±0.42	29	1.46±0.56	34***	1.25±0.62	8

DS, AS, and Δ-4 are plasma dehydroepiandrosterone sulphate, androsterone sulphate and androstenedione, respectively. 11DKS, A, Ae, DHA and 17-OHCS are urinary 11-deoxyketosteroids, androsterone, aetiocholanolone, dehydroepiandrosterone and 17-hydroxycorticosteroids, respectively. 5α/5β is the ratio of androsterone to aetiocholanolone.

Statistical comparisons

British vs Japanese or Hawaiian-Japanese: $^{**}P<0.01$; $^{***}P<0.001$.

Japanese vs Hawaiian-Japanese: $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$.

All the results (except for 5α/5β ratio) are given as logarithms to the base 10.

in which the mean plasma prolactin levels in women with breast cancer have been shown to be similar to that in normal healthy controls [19–24]. Furthermore, abnormalities in plasma prolactin levels are not associated with such risk factors as age at first child, age at menarche, interval between first child and menarche, weight or family history [25]. However, Kwa *et al.* [25] and Henderson *et al.* [26] have reported abnormal prolactin levels associated with family history. There are also reports of abnormalities in nycthemeral rhythms in high risk groups [25, 27], but this has not been investigated so far in studies on geographical risk, and the design of the present study would not have permitted us to detect such an abnormality.

Turning to menopausal and post-menopausal Hawaiian-Japanese women, the characteristics of this group are an increased concentration of oestrone in urine and blood and an increased TSH level. Whether these differences reflect an alteration in the hormonal events of the menopause in Hawaiian women is not clear, but they appear to be unique to the Hawaiian women in that these hormone levels do not differ when British or Japanese women are compared.

In post-menopausal Hawaiians, plasma oestradiol was lower and plasma FSH higher than in British or Japanese women.

The differences in androgen status are very complex. On the one hand, plasma androstenedione and urinary 11-DKS and aetiocholanolone are significantly less in Hawaiian than in British women, and resemble those in Japanese women. However, plasma DS levels are significantly higher than those in Japanese women and resemble those of British women. Our conclusion is that environmental changes alter androgen status in a differential fashion. This is very unlikely to be solely due to changes in body-weight (see Table 1).

The measurement of ovarian and pituitary hormones in this pilot study reinforces the conclusions reached earlier—namely that levels of these hormones do not explain differences in incidence [1–3]. As for the androgens, the results are paradoxical. In British women, subnormal excretion of urinary androgen metabolites is associated with an increased risk of breast cancer [28–30]. In this study the converse is the case; higher plasma and urinary androgen levels are associated with an increased incidence rate. What is even more extraordinary is the finding that in both Japanese and Caucasian women a sub-normal excretion of androgen metabolites is associated with a poor prognosis [31–34] which implies that similar mechanisms operate in both races. It is still possible, therefore, that those Japanese and Hawaiians whose androgen levels are at the lower end of their respective normal ranges are at enhanced risk.

The impression from this pilot study is that changes of environment bring about some alterations in endocrine function, mainly in the androgens. For example, Westernization appears to be associated with a decrease in the $5\alpha/5\beta$ ratio, with an increase in plasma DS, but with no marked change in plasma androstenedione and in urinary 11-DKS. The changes in $5\alpha/5\beta$ ratio are of particular interest in that this is affected by thyroid function; the high and low ratios being associated with hyper- and hypothyroidism, respectively. Corticosteroid levels are lower in Japanese than in British women with the Hawaiians in an intermediate position.

But there is a striking similarity in the levels of the hormones regarded as classical carcinogens. The differences in incidence may therefore be due to factors other than hormones and these may be immunological [35–37] or an inter-relationship between androgen and corticosteroid levels and immune response [38, 39]. Before this conclusion is accepted, much more work is required on endocrine function in children and adolescents in all three races (since early events may be of great importance) and in a more thorough examination of the difference we have found at the menopause.

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Variation in Expression of S_3 Antigen in Human Sarcoma Cell Lines: Influence of Passages and Medium on Generation of S_3*

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Abstract—*An antigen S_3, common to human sarcomas, has been detected by complement-fixation methods in sarcoma cell cultures (JNCI 57:489, 1976). Initial observations suggested that S_3 was present only during passages 1–3 and uniformly absent in older cell lines. This study was undertaken to determine the constancy of expression of S_3 in repeated passages in S_3-bearing cell lines. In addition, the influence of trypsinization and feeding on S_3 production were studied. Cell cultures prepared from 2 fibrosarcomas and chondrosarcoma were studied in detail in this regard. At each passage a sample was taken for cytological examination and another to determine whether S_3 was present. Expression of S_3 antigen was found to be extremely variable and no change in the distribution of cell types, morphologically identified, was noted, regardless of culture conditions and passage. The study confirms that S_3 antigen is most consistently expressed in early passages of human sarcoma cell lines and that this antigen may reappear in passages even if absent for as long as 4 serial passages. The variability of antigen production cannot be attributed to details of the trypsinization procedures used, the presence or absence of cell products in the medium used, or to the selection of specific cell types in the culture with the passage of time.*

INTRODUCTION

IN THE investigation of human tumor associated antigens, tissue culture cell lines have frequently served as the principal sources of antigen. Among the antigens which have been detected in cultured human cells are those related to melanoma [1, 2], sarcoma [3–8], Hodgkin's disease [9] and gliomas [10]. Unfortunately, in most instances antigen expression is quite variable. Often the antigen is expressed only for a short period, during a single passage or several early passages, and then seems to disappear. In this paper, our efforts to study antigen expression over a several month period in human sarcoma cell lines is reported. In this way, it was hoped to gain added insight into the basis for variation in tumor antigen expression under cultured conditions. For the purpose of this study the antigen assayed was S_3, a complement fixing antigen associated with cultures of human sarcomas [11]. Initial studies had suggested that S_3 was present only during the first three passages of sarcoma cell cultures and was absent in older established cell lines. Observations were made with repeated passages to determine the constancy of expression of S_3 in S_3-bearing cell lines. In addition, the influence of trypsinization and feeding on S_3 production were studied.

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MATERIALS AND METHODS

Tissue culture

Sterile tumor samples were obtained from patients undergoing surgery at the Memorial Hospital for Cancer and Allied Diseases, New York. The tissues on arrival at the laboratory were immeditaely processed for growth in tissue culture using RPMI-1640 with 20% heat inactivated filtered fetal calf serum. Details of techniques used to establish

cell cultures have been described in considerable detail elsewhere [12]. All cell lines were tested periodically for mycoplasma by Fogh's method [13] and any line found contaminated was discarded. Cultures were grown at 37°C and fed with fresh medium once every 3 weeks or more frequently if indicated by a marked increase in the acidity of the culture medium.

S_3 antigen assay

The micro-component fixation technique utilized to determine the presence of S_3 antigen in a cell line has previously been reported [11, 14]. In the present study, antigen preparations were made from cell lines during serial passages.

For preparation of S_3 antigen, confluent cells were harvested with sterile rubber policemen and placed in a 20% by volume cell suspension with phosphate buffer saline (PBS). The cells were then frozen and thawed 3 times after which they were sonicated (20 sec/ml) in 1 min bursts. Such preparations were stored at −70°C until use. These were tested with a standard set of unknown S_3 positive and negative sera obtained from sarcoma patients and normal individuals.

For the test, all dilutions were made with veronal buffer saline (VBS), pH 7.4. The sera were diluted with an equal volume of VBS and then inactivated at 56°C for 30 min. Sheep red blood cells (SRBC) were procured from New York City Department of Health. Amboceptor (Difco Laboratories, Inc. Detroit Mich.) was pretitered and freshly diluted for incubation with SRBC. Pooled titered human cord sera served as a source of complement. Each experiment included control wells of antiserum antigen and complement alone to which sensitized SRBC were added to show that none of the test components were themselves anticomplementary and that a positive reading denoted an antigen–antibody reaction.

Experimental modification of culture conditions

To examine the effect of passaging and growth conditions of S_3, cell cultures derived from two fibrosarcomas and one chondrosarcoma were transferred after either trypsinization or scraping and grown in RPMI-1640 with 20% inactivated calf serum. Then, half the cells handled by each method were fed at 2–3 week intervals by complete replacement of medium: the other half by replacement with 50% fresh medium. A sample was taken at each passage to check for S_3 activity and another sample for cytological examination. The experimental design for RFS-75 is illustrated in Fig. 1. Cell lines BCS-72 and LFS-98 were also processed in 4 batches the same way, up to passage 3 and 5 respectively.

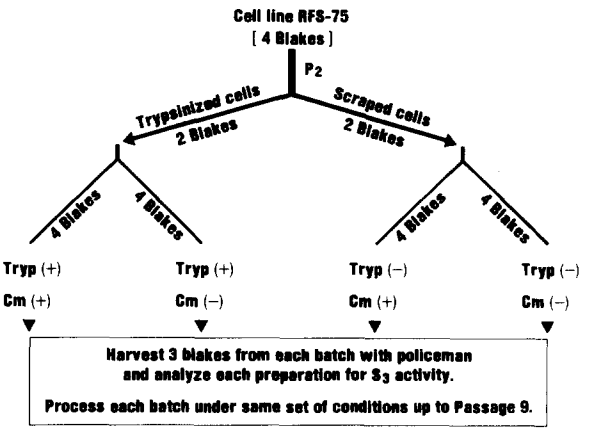

Fig. 1. Experimental design to examine effect of medium and subculturing conditions on S_3 expression. Tryp = trypsinization, Cm = conditioned medium.

Cytology

The fixation and staining techniques for cytological examination of tumor cell lines are described in detail elsewhere [15]. Briefly, the harvested cell pellet from each batch was suspended in 15 ml Earle's balanced salt solution and handled in two ways. The first was the monolayer technique, by which a few drops of the cell suspension were added to a large Leighton tube containing medium with 20% FCS. The cells were allowed to attach and grow on the surface of a glass slide placed in the tube. After incubation for 1–2 days, the cells were fixed in 70% ethyl alcohol and stained with Papanicolaou stain [16]. The second method was a smear technique. The remaining cell suspension was fixed by the addition of an equal volume of 70% ethyl alcohol.

After at least 24 hr fixation at room temperature the cell suspension was centrifuged for 15 min. The pellet was smeared on 4 or 5 albuminized microscope slides. These smears were then fixed in 80% ethyl alcohol for an hour prior to staining with Papanicolaou stain.

RESULTS

To date, 47 successful cultures have been initiated from 78 specimens available for tissue culture (60%). These lines have been

Table 1. Cell lines established and examined for S_3

Diagnosis	Lines	Number	S_3 positive
Osteogenic sarcoma	SOS-10, ROS-11, WOS-19, COS-21, LOS-28 BOS-34, EOS-39, JOS-42, COS-48 KOS-62, COS-91, GOS-132	12	3
Liposarcoma	NLS-8, WLS-13, PLS-35, BLS-50 MLS-64, LMS-76, HLS-80, SLS-121	9	6
Rhabdomyosarcoma	MRS-5, DRS-29, GRS-36, GRS-51 ARS-65, KRS-70, RRS-131	7	3
Fibrosarcoma	KFS-9, PRS-27, RFS-75, GFS-83 SFS-84, DFS-102, KFS-117, RFS-129	8	4
Miscellaneous:			
Chondrosarcoma	RCS-6, BCS-72		
Leiomyosarcoma	LLS-30, PLS-31,		
Synovial sarcoma	GSS-12, RSS-25, CSS-123, SSS-127		
Neurosarcoma	TNS-7, KNS-122, KGS-116	11	5
Total		47	21

examined for S_3 activity over number of passages. As shown in Table 1, 21 lines (44%) were found to contain S_3 in at least one passage. The data for 13 cell lines studied for 3 or more passages for S_3 expression is given in Fig. 2. Of these 13 lines, 11 (85%) expressed S_3 antigen activity during the first 2 passages. By the third passage, only 8 lines were S_3 positive (61%) and in the fourth passage 5 of 10 lines tested showed similar activity (50%). All 5 cell lines retested during a fifth passage, that is, after being in cell culture for about 4 months, had initially been S_3 positive but were now negative. These were respectively 5 lines followed for 6 passages, 4 for 7 passages and 2 for 9 or more transfers. Of the 5 cell lines followed for 6 passages or longer, 3 remained S_3 negative. The exceptions were RFS-75 and KGS-116. Preparations from cell line RFS-75 during passages 6 and 5 did not show the presence of S_3 but did express the antigen again in passage 6, although it then disappeared. Cell line KGS-116 showed similar fluctuations in the expression of S_3. This antigen was present in passage 1, 2, 4, 9 and 11, but absent in intervening passages. Between passages 4 and 9, which were positive, there were four passages of the same line tested which showed no S_3 activity.

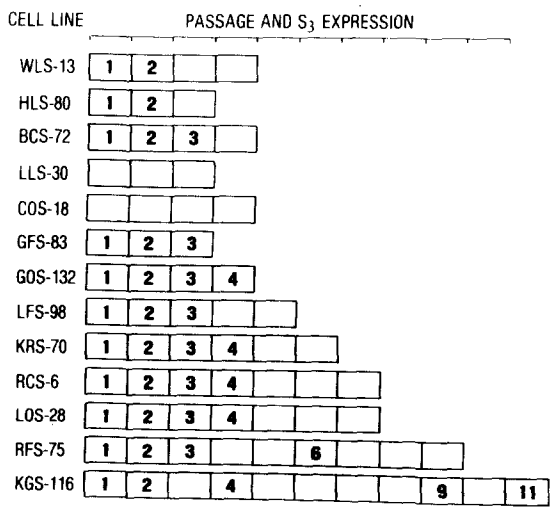

Fig. 2. Variation of S_3 expression with passage. Number denotes the passage in which S_3 was present.

When RFS-75 was studied according to the scheme in Fig. 1, trypsinization and the use of conditioned media both were found not to influence S_3 expression. Preparations from passages 3 and 6 were strongly antigen positive and material from passage 7 weakly positive under all 4 test conditions (Fig. 3). Of interest was the simultaneous appearance and disappearance of antigen in the separate culture bottles examined for S_3. In LFS-98, the antigen was expressed in all batches during passages 1 and 2,

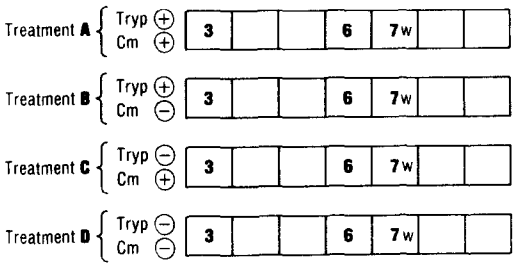

Fig. 3. Antigen expression in RFS-75. Tryp = trypsinization, Cm = conditioned medium, w = weak. Number denotes passage having S_3 activity.

but was completely absent in all cultures of subsequent passages up to passage 5. LFS-98 could not be grown beyond passage 5. Cell line BCS-72 also expressed S_3 antigen in all the 4 batches of passages 1 and 2, but absent in passage 3. Upon cytological examination, no changes in the distribution of cell type present in culture were observed which coincided with changes in antigen expression, conditions of transfers or passages (Fig. 4).

DISCUSSION

While this study confirms that S_3 antigen is most consistently expressed in early passages of human sarcoma cell lines, it also established for the first time that this antigen may reappear in passages even if absent for as long as 4 serial passages. The variability in antigen production cannot be attributed to details of the trypsinization procedures used, the presence or absence of cell products in the medium used, or to the selection of specific cell types in the cultures with the passage of time.

The inconsistent expression of human sarcoma associated antigens in cell lines derived from surgical tumor materials has previously been observed although not directly studied. Priori and co-workers [4] had detected a common sarcoma antigen in early passages only of two cultures of osteogenic sarcoma they tested. In previous work from this laboratory [3], S_1 production was seen to vary with passage although it tended, unlike S_3, to be more readily found in late passages. S_2, a fetal antigen studied in our laboratory [7] was also consistently expressed in early passages only. Moore *et al.* [6] have reported that 10 out of 14 sarcoma cell lines they examined were positive for a common antigen and that the expression of their antigen varied from passage to passage. Bloom [5] has shown that sensitivity of cultured sarcoma cells to the cytotoxic effects of allogeneic antibodies varies with the passage tested. Recently, Cornain *et al.* [2] observed fluctuations in production of melanoma associated antigens which they attributed to changes in culture conditions and growth rate of cells.

The results of present study make it clear that statements describing antigen expression *in vitro* as limited to specific passages or time periods in the life of a cell line cannot be accepted until a sufficient number of appropriate lines have been serially tested for long periods of time. Further, the conditions which determine optimal and consistent expression of tumor antigens in tissue culture systems remain to be defined. Finally, failure to detect an antigen in any single sample of cultured tumor cells should not be accepted as evidence that such a marker is not associated with these cells. If followed diligently, subsequent generations of the same cells may produce readily detectable quantities of antigen.

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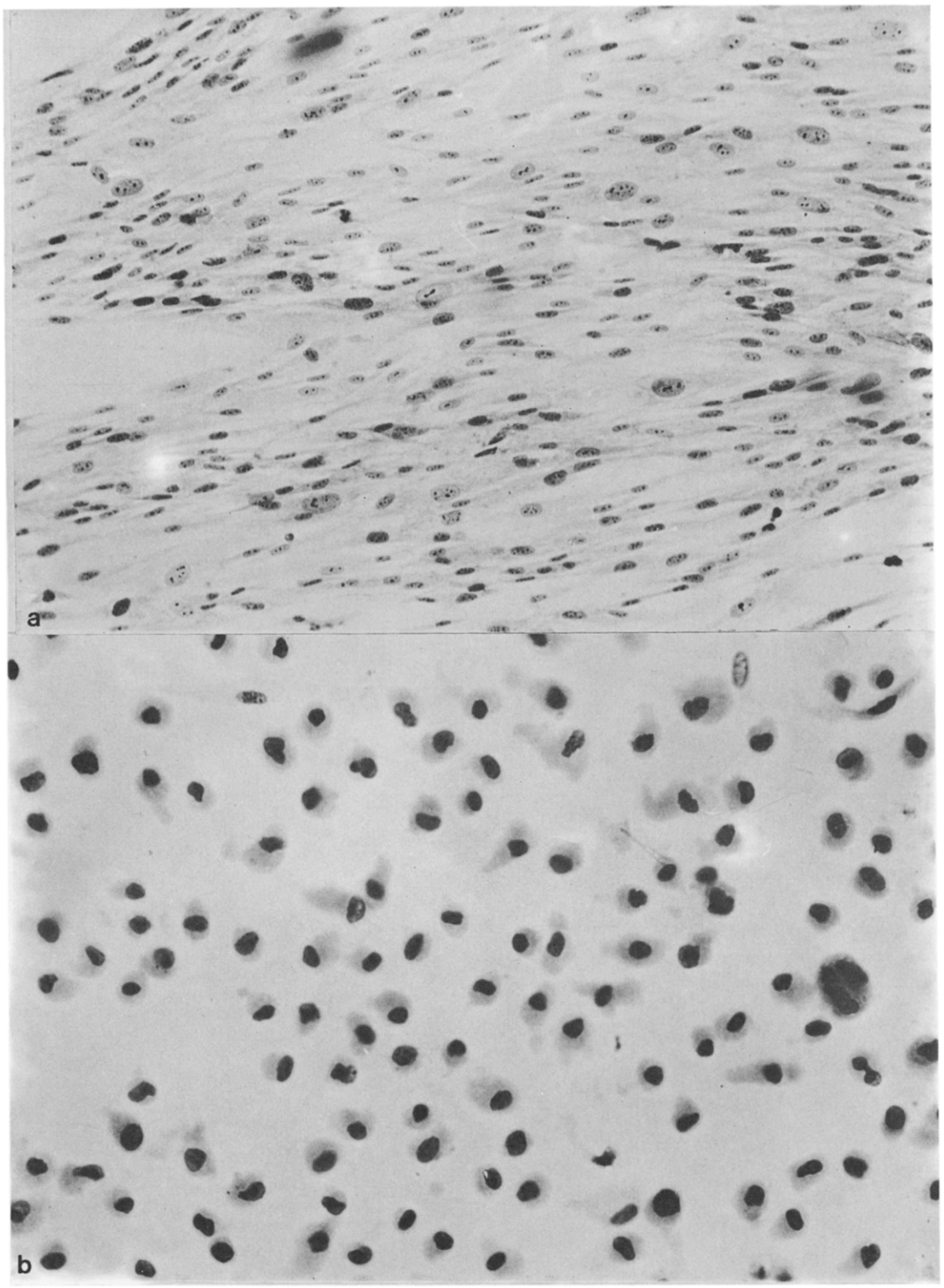

Fig. 4. (*a*) *Stained monolayer culture shows fairly uniform spindle cells with long cytoplasmic processes and sharply demarcated oval nuclei* (*Papanicolaou* × 170). (*b*) *Smear prepared from trypsinized monolayer culture. The cells exhibit moderate pleomorphism. In general, the cytoplasm is oval or elongated and ill defined* (*Papnicolaou* × 350).

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Epidemiologic Characteristics of Urinary System Tumors and Balkan Nephropathy in an Endemic Region of Bulgaria

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Abstract—*In the endemic region of Vratza district, Bulgaria a close epidemiologic similarity was observed between endemic nephropathy (EN) and urinary tract tumors, in particular neoplasms of renal pelvis and ureter. They correlate geographically, affect more women as well as middle aged people, elicit very high incidence rates, reveal a tendency for familial aggregation of cases. In hyperendemic villages age-adjusted annual incidence rates per* 100,000 *were* 43.5 *in men and* 74.2 *in women for tumors of renal pelvis and ureter and* 38.7 *and* 24.6, *accordingly, for tumors of urinary bladder. Urinary tract tumors were often multiple, especially in patients with EN, and as a rule they originate from the uroepithelium. Among all patients with urinary system tumors (UST)* 27.3% *were also registered for EN while UST were found in* 6.5% *of the EN patients. In patients with concurrent occurrence of EN and UST nephropathy was first registered in* 51% *of the cases against in* 18% *for UST.*

The observed epidemiologic similarity between En and urinary tract tumors, their location in urinary system and the fact that they frequently combine is seen as a proof of a close etiologic and pathogenic relationship between the two diseases.

INTRODUCTION

ENDEMIC (Balkan) nephropathy (EN) is a progressive kidney disease observed so far in rural areas of Bulgaria, Yugoslavia and Rumania [1]. In these areas a frequent occurrence of urinary system tumors (UST) has also been encountered in recent years [2–4]. Although intensive investigations on both diseases have been carried out their etiology is not clear and is still a matter of speculation [5].

In this paper, following a report on the geographic correlation between EN and UST occurrence in Vratza district. Bulgaria [6], we present further evidence on their epidemiological correlation and concurrence in some of the patients. Parallel population studies on both disorders are at present unavailable.

MATERIALS AND METHODS

EN cases in Bulgaria have been registered in an area of around 1800 km^2 and a population of about 40,000 people. Our studies were carried out in 23 villages in the same area. The villages were divided into three groups on the basis of EN incidence rates in all endemic villages for the years 1965–1974: *Group A* included 8 villages with highest incidence of EN; *Group B*—7 villages with a moderate EN incidence; *Group C*—8 non-endemic villages, neighbouring the others. All groups of villages were comparable in size, sex distribution, social and working conditions of their population.

Information on EN has been collected from the files of patients registered at the Kidney Disease Clinic, Vratza District Hospital. Cancer cases were sought after a survey of the cancer notification forms, of medical registers in the villages, of medical records in the regional hospitals and in central urologic clinics. This preliminary information was reviewed carefully and cases confirmed on histologic, macroscopic, cytologic, radiologic and/or clinical evidence were analysed. The information on EN and UST was complemented by interviews of the patients or their relatives by members of our investigation team. Annual incidence rates of EN and UST in the years 1965–1974

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Table 1. Distribution by domicile, sub-site and sex of cases with urinary system tumors (UST) in 23 villages of the endemic region of Vratza distract (Bulgaria) for the years 1965–1974

Site No.	Location	Groups of villages A* Male No.	A* Female No.	B† Male No.	B† Female No.	C‡ Male No.	C‡ Female No.
180.0	Kidney, parenchyma	1	–	1	2	1	1
180.1	Kidney pelvis	21	28	6	10	–	3§
180.9	Kidney, part unspecified	3	4	2	3	1	1
181.0	Urinary bladder	24	15	20	7	9	2
181.1	Ureter	4	18	1	7	–	–

*Group A—8 villages with highest incidence of EN and population 75,725.
†Group B—7 villages with moderate incidence of EN and population 71,596.
‡Group C—8 villages with no incidence of EN and population 80,998.
§Two of these women were born and spent 18 and 20 yr after birth in group A villages.

Table 2. Age-adjusted incidence rates per 100,000, mean age and sex ratio of endemic nephropathy (EN), tumors of kidney pelvis and ureter, and tumors of urinary bladder in 23 villages of the endemic region of Vratza district (Bulgaria) for the years 1965–1974

Groups of villages*	Sex	EN Incidence rates	EN Mean age ±S.E.	EN Sex ratio†	Kidney pelvis and ureter Incidence rates	Kidney pelvis and ureter Mean age ±S.E.	Kidney pelvis and ureter Sex ratio	Urinary bladder Incidence rates	Urinary bladder Mean age ±S.E.	Urinary bladder Sex ratio
A	M	315	52.7±1.0	62‡	43.5	54.4±2.2	59	38.7	61.1±2.5	157
	F	506	52.1±0.8		74.2	59.2±1.9		24.6	57.1±3.8	
B	M	96	57.2±1.7	46‡	10.2	57.7±2.3	48	31.1	61.7±2.8	353‡
	F	208	53.4±1.1		21.3	60.3±2.3		8.8	61.2±2.0	
C	M	—	—	—	—	—	—	8.9	71.5±1.4	424
	F	—	—		3.9	51.0		2.1	68.0	

*Group A—8 villages with highest incidence of EN; group B—7 villages with moderate incidence of EN; group C—8 villages with no incidence of EN.
†(M:F)×100—age-adjusted rates.
‡Only figures with this mark are significantly different from 100.

were age-adjusted against a standard world population [7]. Analyses on concurrent affection of people by both EN and UST were done on patients registered in the years 1965–1976. Papillomas and carcinomas of urinary tract were considered together, as it is generally accepted [8].

All quantitative data were evaluated statistically: differences between mean values were tested by the Student's *t*-test while the Chi-square test was used to evaluate the hypothesis of equal risk and a correlation analysis between incidences of EN and UST was made. Values of $P<0.05$ were considered significant [9].

RESULTS AND DISCUSSION

More than 2/3 of all EN patients, registered between 1965 and 1974 in the endemic area of Bulgaria, came from the villages in group A—463 cases and in Group B—182 cases. During the same period of time 185 cases with UST were registered in the studied villages. Their distribution by sub-sites, sex and domicile is given in Table 1. On this basis data on annual age-adjusted incidences, mean age and sex ratio of EN, tumors of kidney pelvis and ureter, and tumors of urinary bladder are represented in Table 2, while Fig. 1 illustrates the age distribution

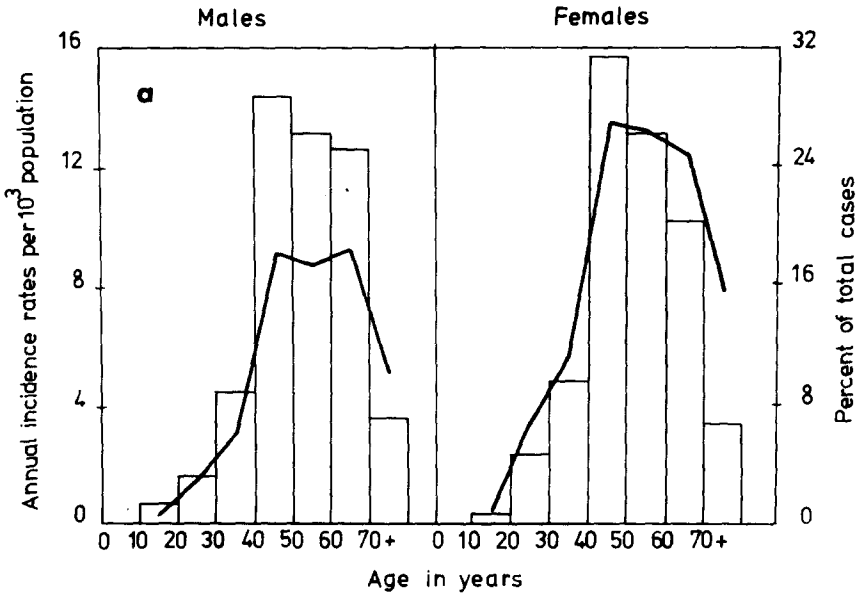

Fig. 1(a). Annual age-specific incidence rates (line) and percentage of distribution (columns) by age and sex of patients (a) with endemic nephropathy (EN).

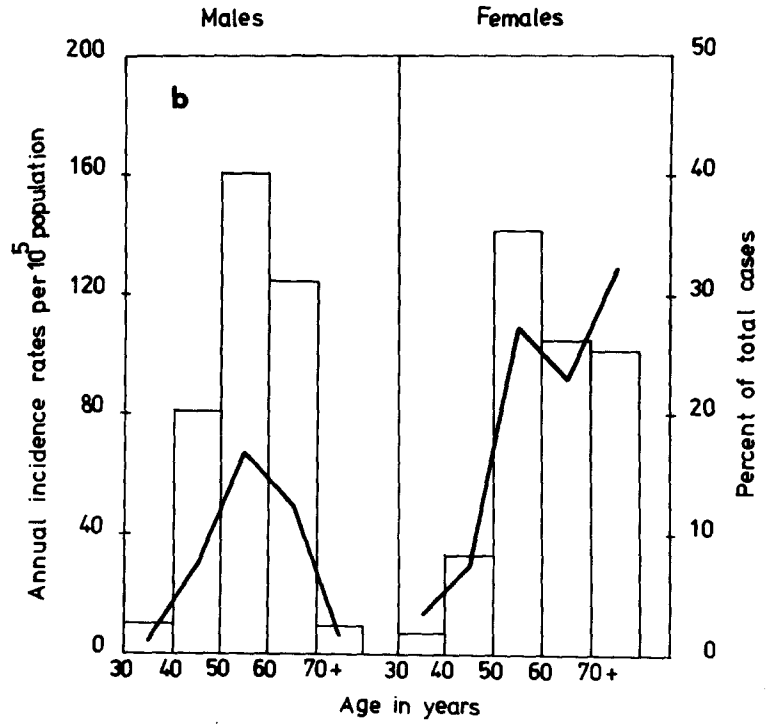

Fig. 1(b). with tumors of kidney, pelvis and ureter.

of the patients with this diseases. Histologically 85% of all urinary tract tumors were from the uroepithelium, 7% were squamous cell carcinomas and 8% were undifferentiated carcinomas.

The results show rather specific characteristics and an interesting relationship between EN and UST:

In the hyperendemic villages UST incidence is in the range of the most common neoplasms recorded for many world populations [8], whereas EN is the leading cause of deaths [6];

The sub-site distribution of UST shows a particularly high occurrence of tumors of kidney pelvis and ureter. Their annual

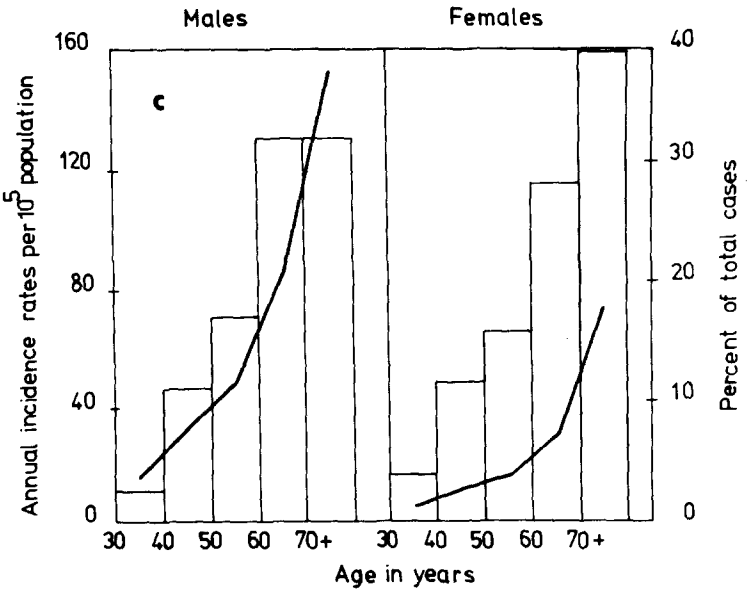

Fig. 1(c). with tumors of urinary bladder for the years 1965–1974. The patients with EN were registered in 8 hyperendemic villages (group A), whereas those with tumors, in 23 villages: 8, with highest incidence of EN; 7, with moderate incidence of EN and 8, with no EN incidence. All villages are situated in the endemic region of Vratza district (Bulgaria).

rates in males and females are 50 and 150 fold higher in comparison with the rest of the country [10–12]. The incidence of parenchyma-cell kidney tumors is close to that in the country as a whole, while urinary bladder neoplasms take an intermediate position. The data, obtained on a population survey, closely resemble observations made on clinical material in Bulgaria [2, 13] and Yugoslavia [3, 4].

It has to be emphasized that on a village basis of a village survey the incidence of EN and UST are highly correlated ($r=0.60$). The mean age of patients with EN as well as of patients with tumors of kidney pelvis and ureter is between 50 and 60 yr. Thus these tumors and even urinary bladder neoplasms affect younger people in the endemic region compared to such tumors outside the endemic area [8, 14–16]. The sex ratio indicates a definite tendency towards predominant affection of women in the area: in the case of urinary bladder cancers, men were more frequently affected but the differences are significant only in group B villages. In the case of tumors of kidney pelvis and ureter more women were affected in all groups although the differences between age-adjusted annual rates by sex are not significant. Nevertheless, in comparison with the existing data on sex ratio of these tumor sites outside the endemic area [8, 10, 14–16], women were relatively much more frequently affected, particularly those from the hyperendemic villages. And finally, EN incidence rates in women are significantly higher than those in men in all groups.

We have also observed multiple urinary tract tumors in some patients. There was a marked correlation between this multiplicity and EN incidence: 15% of the males and 33% of the females affected by UST in group A were with more than one site involved; in group B these cases were 4 and 18% accordingly, and in group C no such patients were observed. Most frequent combinations were tumors of the renal pelvis and ureter, followed by tumors of renal pelvis and bladder. It was also observed that multiple cancers were more frequently present in patients with EN for whom the relative risk of developing such cancers is more than twice as great than for patients with UST but without EN.

Another peculiarity of EN and UST in the endemic area is their concurrence in some patients. Analyses of this combination by sex and domicile are given in Table 3. We found a lower frequency of UST among EN patients in comparison with that published on clinical material. Bulgarian authors [2, 13] found a very high occurence of such neoplasms in EN patients at surgery or post mortem, whereas in the autopsy material of Belgrade clinics this combination

Table 3. Percentage distribution of patients with urinary system tumors (UST) and endemic nephropathy (EN) in relation to all registered for EN and UST patients from 15 villages of the endemic region of Vratza district (Bulgaria) for the years 1965–1976

Groups of villages*	% of UST patients with EN in relation to patients registered for EN			% of UST patients with EN in relation to patients redistered for UST		
	Males	Females	Total	Males	Females	Total
A	7.7	6.7	7.1	29.2	38.0	33.7
B	5.3	4.8	4.9	9.7	27.3	17.0
A+B	7.1	6.1	6.5	21.5	34.7	27.8

*Group A—8 villages with highest incidence of EN.
Group B—7 villages with moderate incidence of EN.

was reported in only 10.5% of the cases [17]. Since our data was obtained from all registered EN cases the observed difference may be assigned to an asymptomatic course, at least for a certain period, of some papillomas of urinary tract in EN patients. Our material also shows that among all patients with tumors of renal pelvis and ureter EN was recorded in 34% of the males and in 36% of the females, against 12 and 29%, accordingly, among the patients with urinary bladder tumors, i.e. in males only EN is significantly more often combined with tumors of the renal pelvis. Finally, in patients with concurrent EN and UST, the nephropathy was registered first in 51% of the patients against 18% for UST. This may be related to various mechanisms, especially different durations of the asymptomatic period of both diseases.

Among the most specific epidemiologic features of EN and UST is a tendency for familial clustering of cases. This has been reported repeatedly for EN [18–20] and recently we have described multiple UST patients in families from the endemic area [21].

A hypothesis for common cause for EN and UST seems most plausible. The familial occurrence of EN and UST suggests their genetical determination but an analysis of the available material does not support such a conclusion [22]. Smoking habits should be also ruled out [23]. Another assumption is that some agent may impair the renal function and structure in people from the area and consequently small amounts of exogenous carcinogens or endogenous metabolites exert their carcinogenic action on the urothelium.

These alternative conclusions should highlight future research in the area.

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Cell Migration and Energy Generating Metabolic Activities

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Abstract—*We have used a "Capillary tube" migration system to investigate the migratory behaviour of peripheral leukocytes obtained from normal human donors and leukemic patients and its relationship to energy-generating metabolic activities.*

Using antimycin A (AA) and 2-deoxy-D-glucose (2-DG) as probes to disturb the metabolic activities that lead to energy production, our preliminary results indicate that the presence of AA (100–800 μg/ml) in the medium fails to influence the migration of normal and leukemic cells. On the other hand, 1–16 mM 2-DG—a potent inhibitor of glycolysis—causes a significant reduction in the migratory behaviour of the normal lymphocytes and lymphocytic leukemic cells. Myeloid cells show a lesser response to this drug. Simultaneous exposure of the cells to both AA (100μg/ml) and 2-DG (1–16 mM), however, results in an augmentation of the inhibitory effects in the case of the leukemic cells. Such an augmentation is particularly noticeable at all concentrations of 2-DG with leukemic lymphocytes.

INTRODUCTION

MIGRATION *in vitro* of leukocytes from human peripheral blood (PBL) has become a major area of physico-chemical and biological research in recent years. Several techniques [1–5] permit quantitative evaluation of the migratory behaviour of individual cells or a cluster of cells. Though simple in appearance, this activity is a complex *mechanical and chemical* phenomenon that involves a number of cellular components and environmental factors [6–9]. The *exquisite* sensitivity of this phenomenon to immunological influences has further attracted the attention of investigators involved in basic as well as applied sciences [10–18].

With the help of the capillary tube migration technique [4], we have been carrying out quantitative studies on the migratory behaviour of normal and malignant human PBL under various physiochemical and immunological environmental conditions. This cellular activity is an energy requiring process and hence is susceptible to chemicals or drugs that interfere with energy-generating metabolic reactions within the cells. In an effort to understand the energy-relationship underlying this activity, we have investigated the influence of some metabolic "probes" on the migratory behaviour of these cells. The present communication deals with data on human PBL migration under the influence of Antimycin A (AA), and 2-deoxy-D-glucose (2-DG), agents that are known to modify intracellular adenosine triphosphate (ATP) pools [19, 20].

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MATERIALS AND METHODS

Cells

Fifteen millilitre aliquots of peripheral blood were obtained from normal human donors and untreated patients with chronic myeloid leukemia (CML) or chronic lymphocytic leukemia (CLL). Additional samples were also obtained from CLL patients who showed blast crisis and had more than 80% blasts in the peripheral blood. Heparin (15 i.u./ml) was used as the anticoagulant.

Normal blood samples were allowed to sediment at 37°C for 60 min, the leukocyte rich supernatant was aspirated and the leukocytes washed twice with sterile normal saline. Purified granulocytes and lymphocytes were obtained with the use of a Ficoll–

Hypaque gradient [21]. Erythrocyte contamination, if any, was removed by subjecting the sample to 0.83% ammonium chloride shock. Final cell pellets were washed twice with normal saline and resuspended in a minimum volume of Eagle's minimal essential medium supplemented with 15% human AB serum (MEM).

Leukemic blood samples were allowed to sediment for 30 min and the supernatants rich in leukemic cells were aspirated. They were then washed twice with normal saline and resuspended in a small volume of MEM.

Leukocyte migration tests

The capillary tube method (1) was used for studying the migration of the leukocyte populations. Aliquots of cell suspension were taken up in 20 μl siliconized glass capillaries (Clay Adams) and sealed at one end with non toxic clay (Seal-Ease, Clay Adams). The sealed capillaries were placed in 13 × 100 mm plastic tubes and centrifuged at 200 ***g*** for 5 min at room temperature to sediment the cells. The volumes of the aliquots were *predetermined* so as to yield 5–6 mm high cell pellets in the capillaries. After cell sedimentation, each capillary was quickly cut slightly below (0.5 mm) the cell pellet–liquid interface and immediately anchored (in duplicate) to the inside edge of the standard plastic migration chamber with a spot of silicone grease. The chambers were then filled with medium alone (controls), or medium along with different concentrations of 2-deoxy-D-glucose (2-DG) or Antimycin A (AA) (experimental). In several experiments, the migration of the cells was studied in medium containing 100 μg/ml AA along with different concentrations of 2-DG. Sealing of the chambers, incubation at 37°C for 24 hr and measurement of the migration areas were carried out as previously reported [22]. Area of cell migration in the control medium was given a migration index (MI) of 1.00 and the effects of 2-DG and AA were expressed in terms of relative MI i.e.

$$\frac{\text{Mean area of migration of four replicates in the experimental chamber}}{\text{Mean area of migration of four replicates in the control chamber}}$$

Each experiment was carried out in duplicate and the samples tested came from 5 normal donors, 5 CML, 4 CLL and 3 CLL patients in blast crisis (Blasts > 80%). The preliminary experiments showed that the variation in the migration area of different types of human PBL in replicate chambers, was in the range of 2–5%. In the present studies, a difference of 10%, in the migration area (in the experimental chambers), was considered significant.

Chemicals

MEM was commercially obtained. Sterile AB serum was collected from normal human AB donors, and was inactivated at 56°C for 30 min before use. 2-deoxy-D-glucose (Serva Fein Biochemica Heidelberg) was dissolved in sterile saline to yield a 100 mM stock solution. Antimycin A, an antibiotic substance produced by *Streptomyces kitazawaensis*, (obtained from Sigma), was dissolved in distilled ethyl alcohol (1 mg/ml). Both the stock solutions were further diluted with the medium to yield the required concentrations.

RESULTS

Antimycin A is a respiratory inhibitor which blocks the electron transport chain [23]. This drug when tested in a range from 100 to 800 μg/ml, did not show a consistent pattern of effect on the migratory behaviour of normal or either type of leukemic cells. Though slight fluctuations in the values of MI (from 0.90 to 1.20) were seen, these had no relationship with the concentrations of AA used in the medium (Fig. 1).

2-DG, a glucose analogue has been reported to be an efficient inhibitor of glycolysis in tissue slices [19] and cell suspensions [27]. In our studies, the 2-DG influence on all the cell types except the CLL blasts, was investigated in 5 separate experiments. In contrast to AA, the presence of 2-DG in the medium inhibited the migratory behaviour of all the cell types studied. Results presented in Fig. 2 show that (a) the inhibitory effect is a function of concentration of 2-DG and (b) amongst the cell types studied, the order of susceptibility seems to be blast cell > CLL cells > normal leukocytes > CML cells.

Figures 3, 4 and 5 depict the modification of 2-DG effects by the simultaneous presence of 100 μg/ml AA, observed in 5 separate experiments. Whereas unfractionated normal leukocytes did not show any significant augmentation of 2-DG effects in the presence of AA (Fig. 3), the myeloid (Fig. 4) and

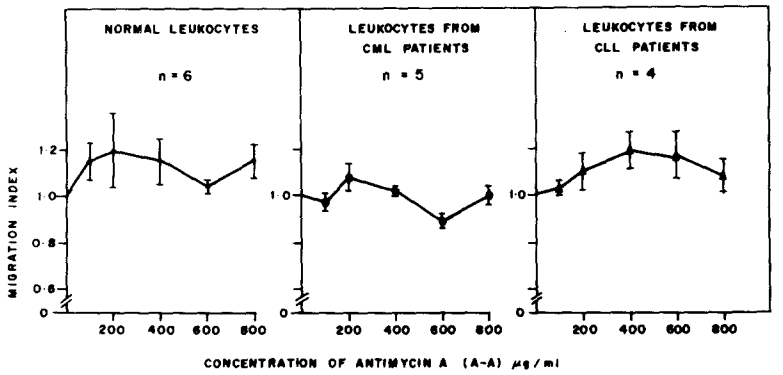

Fig. 1. Migration of different types of leukocytes in the presence of antimycin A (A-A) (mean ± S.E.).

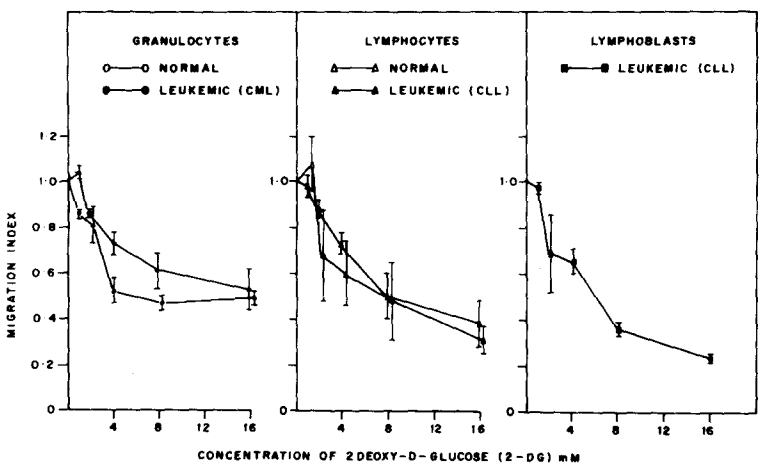

Fig. 2. Migration of different types of leukocytes in the presence of 2-deoxy-D-glucose (2-DG) (mean ± S.E.).

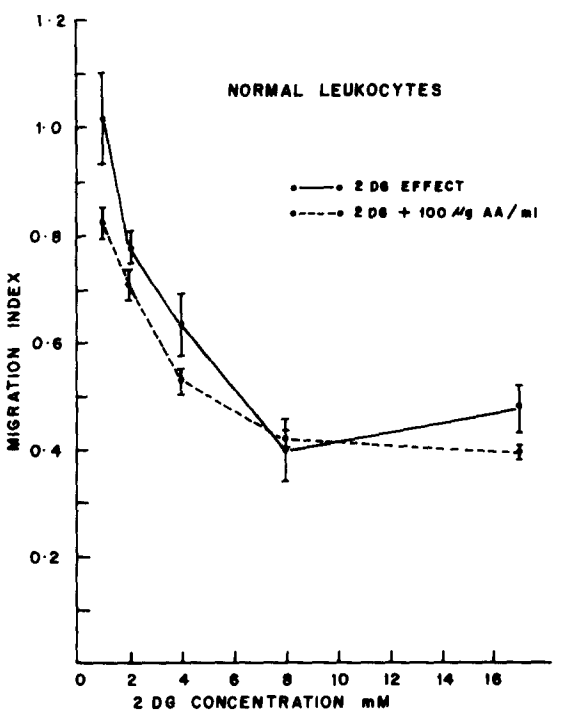

Fig. 3. Modification of the migration-inhibitory effect of 2-deoxy glucose (2DG) by antimycin A (AA).

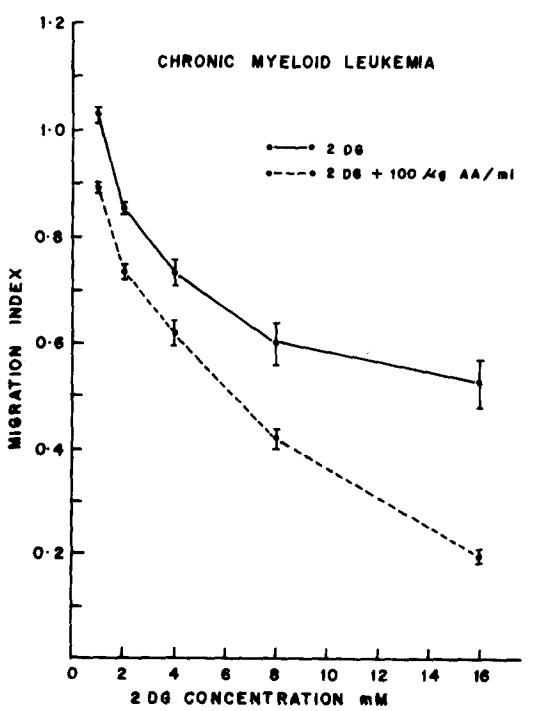

Fig. 4. Modification of the migration-inhibitory effect of 2-deoxy glucose (2 DG) by antimycin A (AA).

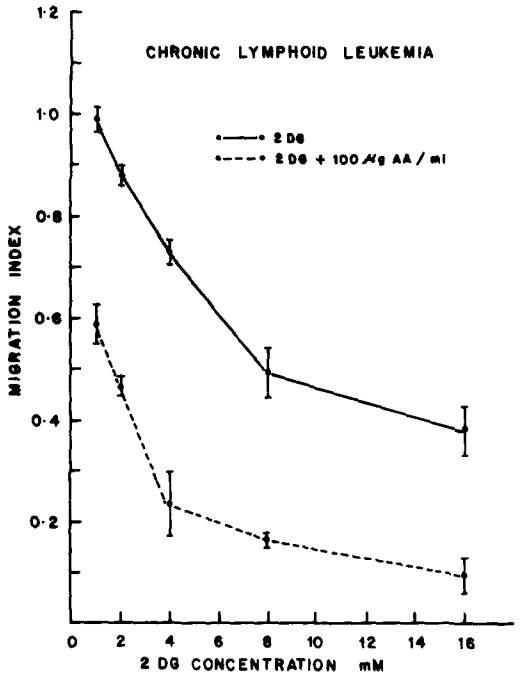

Fig. 5. Modification of migration-inhibitory effect of 2-deoxy glucose (2 DG) by antimycin A (AA).

lymphoid (Fig. 5) types of leukemic cells exhibited a clearly enhanced susceptibility to 2-DG in the presence of AA. This was particularly noticeable for CLL lymphocytes even at 1 mM 2-DG concentration (shift of M.I. Value from 0.98 to 0.59).

DISCUSSION

There have been several investigations concerning the metabolic differences in normal and leukemic blood cells. Attempts have been made to distinguish lymphocytes and granulocytes, normal and leukemic, on the basis of their cytochemical reactions [24], aerobic and anaerobic glycolysis [25–27] and respiratory rates [27, 28]. These studies indicate that normal lymphocytes and granulocytes have an energy metabolism similar to their leukemic counterparts [28]. Further, such studies have also shown the predominance of glycolytic activity in leukocytes [29] as judged from a cellular susceptibility to 2-DG.

Our results indicate that the capillary tube migration system can be used to assess the differential effects of metabolic probes, random motility of leukocytes providing a rapid, reliable and reproducible indicator. These results from leukocyte migration in the presence of 2-DG fall in line with the past evidence. The higher susceptibility of migration of the lymphoid cell types to 2-DG also agrees with the finding that lymphocytic cells have a high rate of anaerobic glycolysis [29] and presumably depend on this primarily for their energy requirements.

The flexibility of leukocyte energy metabolism is well known, since blocking of respiration has been reported to cause an increase in glycolysis and vice versa [30]. Augmentation of 2-DG mediated migration-inhibition in the presence of AA—respiratory inhibitor—indicates the prevention of such a shift so as to make the cells more susceptible to the glucose analogue. The CLL lymphocytes show such augmentation even with the lowest 2-DG concentration used, in spite of the presence of 5 mM glucose in the medium.

Metabolic blocking of the glycolytic pathway [30–33] has been reported to affect leukocyte functions like phagocytosis and permeability. Results presented here indicate leukocyte migration also to be a sensitive indicator of similar metabolic disturbances.

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Correlation Between Immunosuppression to Tumorigenesis. 1. Humoral Immunosuppressing Factor

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Abstract—*Factors that alter cellular immunity, macrophage and lymphoid cell functions can be obtained from sera of animals bearing proliferating tumors. Rats bearing syngeneic mammary adenocarcinoma (R 3230) AdCa) and CFW mice bearing syngeneic spontaneous mammary adenocarcinoma have been examined. Peritoneal macrophages and spleenocytes cytotoxicity against tumor cells, and the effect of serum on incorporation of ^{3}H-thymidine into normal spleenocytes DNA in response to phytohemagglutinin (PHA) were assessed at time intervals during tumour growth.*

1. *If drawn early, during the* 1st *to the* 6th *week, after s.c. injection of tumor cells enhanced, but if drawn later,* 7th *to expiration week, the serum suppressed the response of normal spleenocytes to PHA.*

2. *Macrophages and spleenocytes from normal animals had no effect, those from animals bearing a subcutaneous progressively growing syngeneic tumor enhanced proliferation, whereas those from animals immunized against the tumor were cytotoxic against syngeneic tumor cells.*

3. *Cell-free supernatants harvested from mixed cultures at ratios between* 10:1 *to* 8.3×10^3 *of normal spleenocytes, or spleenocytes from immune animals to syngeneic tumor cells enhanced, whereas supernatants from mixed cultures of spleenocytes of tumor-bearing animals to syngeneic tumor cells suppressed the response of normal spleenocytes to PHA.*

4. *Cell-free supernatants harvested from mixed cultures at* $1:10^4$ *ratio of syngeneic tumor cells with spleenocytes of animals early (during the* 1st *to* 4th *week) after s.c. injection of tumor cells enhanced, but those from mixed cultures of cells suppressed the response of syngeneic normal spleenocytes to PHA.*

INTRODUCTION

BLOOD from patients and animals bearing tumors contain several factors that modify the response of the host to a growing neoplasm. The relation between these factors and tumor burden could be of diagnostic and prognostic value, and warrant further investigations.

Immunosuppression accompanies tumor growth in both patients and animals [1–4], and immunosuppressive factors impair immunocompetence as measured by decreased reactivity of host's lymphocytes to mitogenic stimulation [5–8]. Cytotoxic activity of blood lymphocytes from cancer patients for cultured cells derived from human tumors has been considered to be indicative of the presence of a tumor-host response [9, 10]. "Blocking factors" also have been demonstrated in several host-tumor systems [11].

Many cancer patients and tumor-bearing animals, at least in the early stage of the tumor proliferation, possess "potentiating" [12–14] activities which render the infiltrating lymphocytes capable to stimulate rather than suppress proliferation of tumor cells [15]. These activities are mediated by immunoglobulins [16], antigens shed from cell membrane [17, 18] or tumor-specific antigen–antibody complexes [19, 20].

To be of clinical value, the factors by which humoral and cellular immunity control the rate of tumor proliferation must be quantitatively related to tumor burden. The present studies examine the *in vivo* correlation between humoral immunosuppression immunostimulation and cell-mediated cytotoxicity to tumor burden. They demonstrate a correlation between the presence of immuno-

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stimulating–immunosuppressing agents in cell-free supernatants harvested from *in vitro* mixed cultures of spleenocytes with syngeneic tumor cells. The pattern of immunostimulation–immunosuppression depended on the source and ratio of spleenocyte to tumor cells.

MATERIALS AND METHODS

Animals and tumors

Inbred female CFW white mice, 6–8 weeks old, from our colony, were used. The syngeneic mouse tumor studied was a mammary adenocarcinoma (CFW-AdCa) that arose spontaneously in our CFW colony. It was maintained for 35 generations by serial s.c. inoculation of 1×10^4 living cells suspended in 0.2 ml of Hanks' basal salt solution (HBSS).

Inbred female Fischer rats, 12–16 weeks old, from our colony, were used. The tumor was DMBA-induced mammary adenocarcinoma (R 3230 AdCa). It was maintained for 27 generations by serial s.c. inoculation of 1×10^8 living cells suspended in 0.5 ml of HBSS. The injection site was palpated for the presence of tumor every two days, and growth rates were measured with a caliper.

Target cell cultures

CFW-AdCa and R3230 AdCa monolayers were established *in vitro* as described under condition TC "a" [19]. After treatment of the excised tumors with collagenase (10 μm/ml) supplemented with 1000 μm of deoxyribonuclease/ml (DNAase I Sigma) the cells were washed twice. They were subcultured *in vitro* for 3–4 passages before use. The cell lines were constantly tested for tumorigenicity.

One hundred percent of syngeneic animals injected s.c. with 1×10^5 cultured tumor cells developed tumors resembling the primary tumors in their growth pattern and histological characteristics.

Sera from immune and tumor-bearing mice and rats

Different groups of tumor-bearing mice and rats, injected with 1×10^4 CFW-AdCa (or 1×10^8 R3230 AdCa) were bled on the 2nd, 6th, 9th, 12th, 15th, 17th, 22nd, 27th, 34th and 45th day after isograft. CFW-AdCa or R3230 AdCa hyperimmune sera were obtained from CFW mice or Fischer rats injected 4 times at weekly intervals with 5×10^5 neuraminidase-treated and glutaraldehyde-fixed tumor cells [20]. All the sera were heat inactivated at 56°C for 30 min and stored in separate vials at −20°C until required. The sera were diluted 1:5 with Eagle's minimum essential medium (MEM) and filtered through 0.22 μm Millipore® filters before use.

Preparation of spleenocytes

Spleens from normal, tumor-bearing and immune animals were dissected at time intervals, minced with sterile forceps, and gently teased through a 80-gauge sterile stainless steel screen. The cells were washed 3 times in HBSS, suspended in Eagle's MEM supplemented with 15% fetal calf serum (FCS) and incubated 24 hr in cotton stoppered tissue culture bottles at 37°C in a humidified atmosphere of 5% CO_2 and 95% sterile air [21]. The non-adherent cells were collected, washed 3 times in HBSS, and suspended in Eagle's MEM at a concentration of 5×10^5 viable cells/ml. Cellular viability assessed by trypan blue exclusion, was greater than 95% in all experiments.

Macrophage cultures

Macrophages were obtained from normal CFW mice or rats, CFW mice or rats bearing a progressively growing subcutaneous CFW-AdCa or R3230 AdCa, or from CFW-AdCa or R3230 AsCa mice or rats immunized against the syngeneic tumor. Two millilitres (or 10 ml) thioglycollate was injected i.p. into each mouse (or each rat) and 4–5 days later, the animals were sacrified and their peritoneal exudate cells (PEC) were harvested by being washed with HBSS containing 2 units heparin/ml. The cells were centrifuged and resuspended in Eagle's MEM; 1×10^7 cells were plated in 100×20 mm plastic Petri dishes and placed in a humidified 37°C incubator containing 5% CO_2 atmosphere.

Five days after the i.p. injection with thioglycollate, the harvested PEC consisted of about 85% large, vacuolated mononuclear cells with typical macrophage morphology. The remaining 13–15% consisted of lymphocytes and some granulocytes. Washing the non-adherent cells with Eagle's MEM 1 hour after initial plating in culture dishes removed the lymphocytes. Granulocytes are short-lived cells and were removed when adherent PEC cultures were refed on the 5th culture day [22], at that time, all the remaining adherent cells were capable

of phagocytizing carbon [23], had the typical macrophage morphology and were used in the present studies.

Biological activities of cell-free supernatants and sera

The various non-adherent cells from the spleen cell cultures described earlier were added to tumor cell cultures at a ratio of 10^3:1, approximately 1×10^6 tumor cells per flask in 20 ml of Eagle's MEM. After 24 hr incubations, the supernatants from the mixed cultures and from incubations containing the non-adherent cells or the tumor cells each alone (control) were collected, centrifuged at 6000 ***g*** for 10 min and passed through a 0.22 nm Millipore® filter. The cell-free supernatants were then tested as follows:

(*a*) *Macrophage activation.* The supernatants were added to 4-day old cultures of macrophages from syngeneic normal, tumor-bearing or immunized animals. After 48 hr, the macrophages were collected, washed with Eagle's MEM and harvested with the aid of a soft, wide-tipped rubber policeman. Cell counts and viability were determined, they were suspended in cold HBSS, then examined for cytotoxic activity against syngeneic tumor cells.

(*b*) *Effects on response of normal spleenocytes to phytohemagglutinin (PHA).* Spleenocytes were obtained from syngeneic normal mice or rats. They were passed through sterile cotton-wool columns and eluted with Eagle's MEM. After 3 washings, the cells were counted and viability assessed by the Trypan blue dye.

In culture tubes, aliquots of 10^5 normal spleenocytes in 1 ml of Eagle's MEM supplemented with 15% FCS plus 100 u/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine. All tubes except the unstimulated control contained 10 μg of purified PHA. Experimental tubes contained aliquots of supernatants equivalent to 10 μg protein. Additional control tubes contained a concentration of reference pooled normal mouse or rat serum equal to the greatest concentration of experimental protein tested. The tubes were then maintained in a 5% CO_2 humidified environment at 37°C for 50 hr then pulsed by the addition of 1 μCi of ^{3}H-thymidine for 18 hr. Before harvesting, viability of the cells was reassessed. In these experiments viability did not differ in control or experimental groups. The cultures were harvested and DNA synthesis was determined as previously described [24].

The data were computed as percentage of suppression (−) or stimulation (+).

$$\% \text{ Suppression} = \frac{\text{Cpm experimental tubes with PHA} - \text{Cpm control tubes without PHA}}{\text{Cpm control tubes with PHA} - \text{Cpm control tubes without PHA}} \times 100$$

(*c*) *Quantitative assay of cell-mediated cytotoxicity* in vitro. Tumor cells growing *in vitro* at confluency were labeled for 24 hr with 0.25 μCi of ^{51}Cr per ml of Eagle's medium. Before the assays, the cultures were washed with HBS to remove all unbound radioactive material. The labeled tumor cells were harvested by short trypsinization (0.25% trypsin for 1 min) and suspended in the supplemented Eagle's MEM.

Routinely, single-cell suspension of target cells were mixed with macrophages (or spleenocytes) at various ratios and rotated for 1 hr on a platform at room temperature, plated in culture dishes and incubated for 5 days. Twenty-four hours after plating, the cultures were refed, on day 5 they were washed twice with HBSS to remove all radioactivity not associated with adherent, presumably viable target cells. The adherent cells were lysed with 1 ml of 0.5 N NaOH and washed twice with HBSS. The lysate and the HBSS washes were combined and radioactivity was monitored in a gamma counter. Each sample was counted three times for 5 min and percentage cytotoxicity was computed.

$$\% \text{ Cytoxicity} = \frac{\text{Cpm in adherent cells alone} - \text{Cpm in tumor cells with macrophages}}{\text{Cpm in adherent cells alone}} \times 100$$

RESULTS

Briefly, the present studies were aimed to identify the biological activities of agent(s) released as a result of tumor cell proliferation. The first groups of experiments were performed to determine whether s.c. injection of syngeneic tumor cells will produce activities which alter lymphoid tissue, i.e. spleenocytes functions.

1. *Stimulation-suppression of the response of syngeneic spleenocytes to phytohemagglutinin (PHA) with serum from animals isografted with mammary adenocarcinoma*

In agreement with earlier results [28], PHA stimulated the incorporation of ^{3}H-

Table 1. Correlation between tumor size, and effect of serum drawn after isograft on response of normal spleenocytes to phytohemagglutinin (PHA)

Serum drawn days after isograft	Non-stimulated			PHA-stimulated				Tumor size (mm)
	Alone	With serum	% Control	Alone	With serum	% Control	Percentage*	
0	435±14	565±17	129.8	51585±508	70568±617	136.8	+ 7.0	Nil
5	478±16	683±21	142.8	51628±493	96338±743	186.6	+43.8	Nil
10	689±19	942±27	136.7	39784±356	83467±685	209.8	+73.1	Nil
15	897±25	877±26	97.7	48935±396	87643±705	179.1	+81.4	Nil
20	723+21	745±22	103.0	44876±326	67705±513	153.3	+50.3	3.1
25	972±47	764±26	78.6	45932±378	42395±318	92.3	+13.7	3.7
30	636±18	587±18	92.2	52686±462	48682±414	92.1	+ 0.2	4.1
				Suppression				
35	685±24	664±24	96.6	51835±524	33123±326	63.9	−32.7	7.6
40	815±35	713±22	87.6	51965±437	11069±218	21.3	−66.3	13.2
45	773±25	709±22	91.7	50047±436	11160±247	22.3	−69.4	17.8
50	924±24	815±27	88.2	39764±326	2068± 96	5.2	−83.0	23.1

The results are presented as the average ± S.D. of 4 separate determinations.
*The percentage is equal to = % of control of stimulated − % of control of non-stimulated.
(+) is stimulation. (−) suppression.
Control spleenocytes were exposed to the same experimental conditions using Eagle's medium in place of the serum used.

thymidine into lymphocytes and spleenocytes. The magnitude of this effect varied with the type of cells and was influenced by several factors added to the incubation medium. Therefore, aliquots of serum drawn at zero and at time intervals from animals injected with mammary adenocarcinoma cells were examined.

(a) Correlation between tumor size and effect of serum drawn from rats isografted with R3230 AdCa on response of normal syngeneic spleenocytes to PHA. After inoculation of R3230 AdCa cells into syngeneic unconditioned rats, the serum drawn during the early period, between 0 and 30th day, enhanced whereas, sera drawn at later periods reduced the response of normal syngeneic spleenocytes to PHA. The data summarized in Table 1, indicate a correlation between the serum suppressing effect and tumor size. Stimulation was observed with serum drawn from time zero and increased to a peak between the 10th and 20th day. At later period it progressively decreased to nil. Sera obtained between the 30th to expiration day produced suppression which progressively increased. During this latter period, the tumor grew to reach approximately 60 mm in diameter.

(b) Correlation between tumor size and effect of serum drawn from CFW-mice isografted with CFW-AdCa cells on response of normal syngeneic spleenocytes to PHA: After inoculation of CFW-AdCa into syngeneic unconditioned CFW-mice stimulation

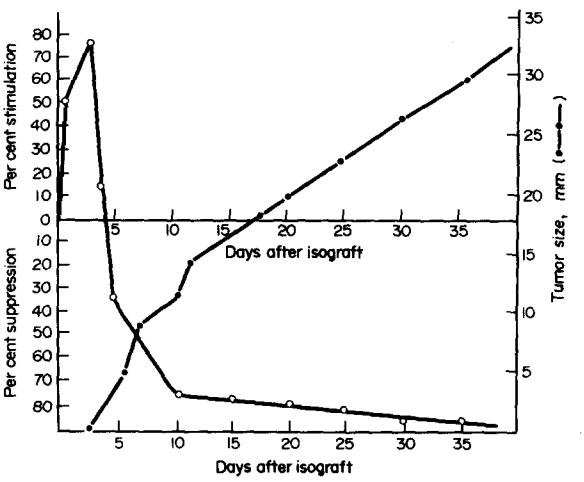

Fig. 1. Effect of murine serum on syngeneic murine spleenocytes response to phytohemagglutinin during tumor growth. Response to phytohemaglutinin (—○—○—), Tumor size in mm (—●—●—).

was observed with serum drawn from zero time, increased to peak between 3rd and 4th day, and decreased to nil on the 5th day. On the fourth day all the animals had palpable nodules of approximately 2 to 3 mm in diameter. Sera obtained between the 5th day to expiration day suppressed the response of normal syngeneic spleenocytes to PHA. During the latter period tumor grew progressively from 2.5 to 33 mm in diameter. The data depicted from the two curves in Fig. 1 indicate correlation between the serum suppressing activity and tumor size.

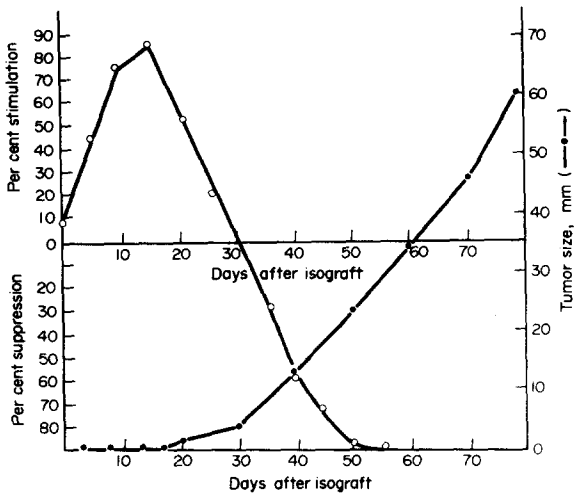

Fig. 2. Effects of rat serum on response of syngeneic spleenocytes to phytohemagglutinin (PHA) during tumor development. Response to PHA (○—○) and tumor size in mm (●—●).

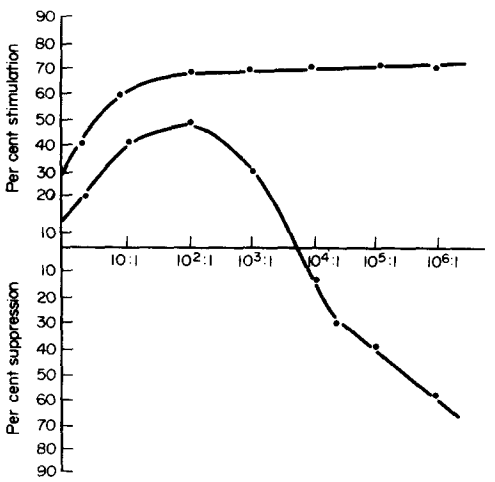

Fig. 3. Effect of supernatants from mixed cultures at various ratios of syngeneic rat spleenocytes to R3230 AdCa tumor cells on response of normal syngeneic spleenocytes to phytohemagglutinin. R3230 AdCa cells with spleenocytes from (A) tumor-bearing and (B) immunized rats.

(c) Correlation between tumor size and effect of serum drawn from rats isografted with R3230 AdCa on response of normal syngeneic spleenocytes to PHA. The data summarized by the two curves in Fig. 2, indicate a correlation between the serum suppressing effect and tumor size. Stimulation was observed with sera obtained from time zero and increased to peak between 15th and 20th day and decreased to nil on the 29th day. Data obtained after the 30th day suppressed mitogenic stimulation of normal syngeneic spleenocytes. During this latter period, the tumor grew progressively from 3 to 60 mm in diameter.

2. *Stimulation–suppression of the response of syngeneic spleenocytes to PHA with cell-free supernatants from mixed cultures of sensitized spleenocytes and syngeneic tumor cells*

To establish the origin of serum stimulating–suppressing activities, normal, sensitized spleenocytes and tumor cells, each alone and mixed cultures were incubated at 37°C in 5% CO_2 atmosphere for 5 days. Cell-free supernatants from mixed cultures of spleenocytes from normal syngeneic animals and R3230 AdCa cells had no effect, if harvested from mixed cultures at low ratios (10:1 to 10^3:1) spleenocytes from syngeneic rats bearing progressively growing tumors and R3230 AdCa cells, the supernatants enhanced, but from higher ratios (10^4:1 to 10^6:1) inhibited the response of normal syngeneic spleenocytes to PHA (curve A, Fig. 2). Cell-free supernatants harvested from mixed cultures of spleenocytes from immunized animals with R3230 AdCa cells enhanced (curve B, Fig. 3) the response of normal syngeneic spleenocytes to PHA. The magnitude of the effect varied with the ratio of spleenocytes to tumor cells. Not shown in the figure, supernatants from cultures of spleenocytes alone had no effect, whereas supernatants from cultures of R3230 AdCa cells alone produced weak but statistically significant ($P<0.005$) suppression of the response of normal syngeneic spleenocytes to PHA. Not reported here, similar patterns were obtained with cell-free supernatants from mixed cultures of spleenocytes from CFW-mice bearing progressively growing CFW-mammary AdCa.

Therefore, these experiments demonstrated that cell-free supernatants from mixed cultures of sensitized spleenocytes, i.e. spleenocytes from immunized animals (at all ratios) and from animals bearing progressively growing tumors (at low ratios), with syngeneic tumor cells stimulated the response of normal syngeneic spleenocytes to PHA ($P<0.001$). Since supernatants from cultures of the spleenocytes had no effect, but supernatants from cultures of tumor cells alone had statistically significant suppressing activity, it is suggested that the suppressing agent(s) may be released by the tumor cells. The origin of the stimulating agent(s) must await further studies.

3. *Evolution of cell-mediated cytotoxic activities against mammary adenocarcinoma*

Cytotoxic activity of lymphoid cells from hosts bearing neoplasms for cultured cells

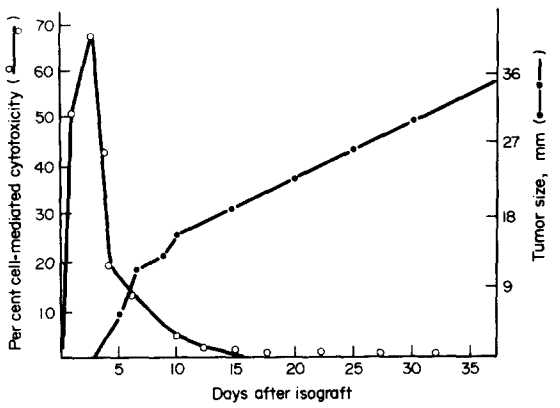

Fig. 4. Evolution of cell-mediated cytotoxicity against R3230 AdCa. Spleenocytes were taken from tumor-bearing rats at time intervals during the progressive growth of the adenocarcinoma. Each reading represents the average of 4 separate determinations. The variation between the determinations varied less than 5% with a $P<0.001$.

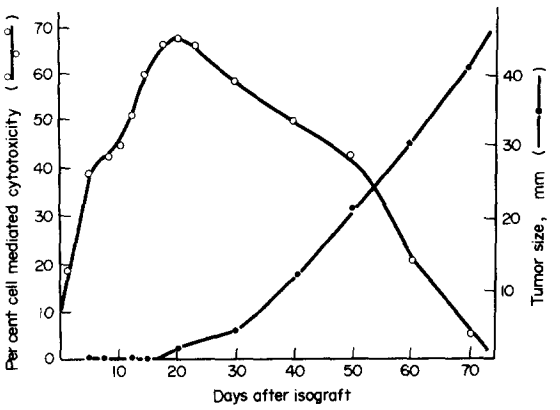

Fig. 5. Cell-mediated cytotoxicity against CFW-AdCa cells. Spleenocytes were taken from tumor-bearing syngeneic animals at time intervals during the progressive growth of the tumor in CFW-mice. Each point represents the average of 4 separate determinations. The variation between each determination varied less than 5% with a $P<0.001$.

derived from the host tumor indicate the presence of a tumor-associated host response. The following experiments are aimed at the evolution of cell-mediated cytotoxicity directed against syngeneic tumor cells during growth of the tumor.

(a) Cell-mediated activity against rat mammary R3230 AdCa cells: the results depicted by curve "a" Fig. 4, indicate that before inoculation of the tumor cells, normal syngeneic spleenocytes possessed statistically significant 10% ($P<0.05$) cytotoxic activity against R3230 AdCa. This cytotoxicity increased to reach a peak between the 19th and 21st day after isograft, then decreased to a very low level. In these experiments solid tumors became palpable between the 19th and 21st day after injection of the tumor cells (curve b, Fig. 3) a period where maximum cytotoxic activity was attained. Spleenocytes from either normal or R3230 AdCa-bearing rats had no effect against murine CFW-AdCa cells.

(b) Cell-mediated cytotoxic activity against murine mammary CFW-AdCa cells. The data presented by curve "a" Fig. 4, indicated that before isograft normal syngeneic spleenocytes had statistically non-significant cytotoxic activity against CFW-AdCa cells. This cytotoxic activity increased sharply after s.c. injection of CFW-AdCa cells to reach a peak on the 3rd day, then decreased to nil on the 16th day. Solid tumors became palpable on the 3rd or 4th day after the isograft (curve b, Fig. 5), it is the period at which maximum cytotoxicity was observed. The size of the tumor increased progressively to reach 20 mm in diameter on the 16th day, where spleenocytes became non-effective against syngeneic CFW-AdCa cells. Spleenocytes, either from normal or from CFW-AdCa-bearing animals had no effect against R3230 AdCa.

Therefore, the magnitude of spleenocyte cytotoxic activities correlate inversely with the size of the tumors. It is suggested that if tumor size continues to increase at the peak value of cytotoxic activity, it is indicative of failure of the host cell-mediated immunity to interfere with the growth of the tumor.

4. *Correlation between serum blocking and suppressing activities with tumor size*

The patterns of serum immunostimulation–immunosuppression, and spleenocyte cytotoxic activity showed direct correlation between suppression and tumor size and an inverse relation with spleenocyte cytotoxic activities. The following experiments examine the relation between serum "blocking" activities and tumor size. The data summarized in Table 2, indicated a correlation between number of tumor cells isografted with tumor size. The changes in tumor size were accompanied with changes in both serum "blocking" and suppressive activities. The magnitude of increase in serum blocking or suppressive activities varied directly with number of tumor cells injected.

Therefore, these experiments showed patterns of serum biological activities which accompanied progressive growth of the tumor. Although the patterns suggest similarities between serum "blocking" and suppressive activities, the data do not permit differentiation of the two serum activities. Whether

Table 2. Correlation between tumor size, serum blocking and suppressive activities

Number of tumor cells injected	Days after injection	Tumor size (mm)	Serum blocking activity (%)	Serum suppressive activity* (%)
CFW-AdCa				
1×10^4	5	Nil	−22±3.3	+68±6.4
1×10^4	20	4±0.6	− 8±0.5	+ 5±0.4
1×10^4	35	17±1.4	69±6.5	−64±5.6
1×10^6	4	3±0.1	5±0.6	+10±1.2
1×10^6	9	10±1.6	36±5.9	−65±6.8
1×10^6	15	19±1.2	84±4.7	−77±7.5
1×10^8	6	14±1.9	47±3.7	−69±6.7
1×10^8	9	24±1.7	94±7.6	−92±7.8
R3230 AdCa				
1×10^5	15	Nil	−35±3.1	+82±7.2
1×10^5	35	9±0.7	36±2.2	−44±3.6
1×10^5	50	24±4.3	61±5.7	−35±2.7
1×10^7	7	4±0.3	48±2.9	−28±2.5
1×10^7	12	12±1.9	79±5.8	−72±6.5
1×10^9	4	10±0.8	52±5.0	−68±6.1
1×10^9	9	32±2.2	94±6.1	−95±7.8

*(+) indicates stimulation. (−) indicates suppression. The above data represent the average±S.D. of six separate experiments.

caused by the same or different agent(s), the results indicated that the serum activities reflect changes in tumor size.

5. *Correlation between macrophage functions and tumor size*

Macrophages play a role in the control of tumor development, but specificity of the reactions involved is at present unclear. Whether from normal CFW-mice or from rats, peritoneal macrophages had statistically non-significant effects on response of syngeneic spleenocytes to PHA. On the other hand, macrophages from tumor-free immune animals enhanced, whereas macrophages from hosts bearing progressively growing tumors suppressed the response of normal syngeneic spleenocytes to PHA. The data summarized in Table 3, indicated that the magnitude of suppression varied also with number of inoculated tumor cells and time after isograft. Macrophage suppressing activities correlated with the number of cells in the isograft and with tumor size. It is suggested that during progressive tumor development, macrophages suppress spleenocyte functions. The present data do not permit any conclu-

Table 3. Macrophage activities and tumor size

Tumor (animal)	Days after tumor cell inoculation	Tumor size (mm)	Macrophage suppression (−) or stimulation (+) (%)
CFW-mice normal	—	—	+ 5±0.7
CFW-mice immunized	—	—	+82±7.2
CFW-mice inoculated			
AdCa 1×10^4	5	Nil	+61±5.3
1×10^4	20	4.5±2.3	−12±1.1
1×10^4	35	17 ±3.4	−72±6.8
1×10^6	4	2.4±0.1	+15±1.1
1×10^6	9	11 ±1.6	−69±5.4
1×10^6	15	18 ±1.3	−72±6.9
Fischer rats normal	—	—	+ 8±0.5
Fischer rat immunized	—	—	+76±6.3
Fischer rats inoculated			
R3230 AdCa 1×10^5	15	Nil	+68±5.7
1×10^5	35	10±3.0	− 8±0.7
1×10^5	50	23±4.3	−70±6.1
1×10^7	3	Nil	+82±6.5
1×10^7	7	4±0.5	−34±2.7
1×10^7	12	15±1.7	−83±6.3
1×10^9	2	2.7±0.8	−11±0.9
1×10^9	4	11 ±0.8	−71±6.8
1×10^9	9	32 ±2.8	−96±6.4

The above data represent the average±S.D. of seven separate experiments.

Table 4. Influence of tumor size and surgical tumor removal on serum activities

Animal-tumor	Days after tumor cell inoculation	Tumor size (mm)	Blocking (%)	Serum activities suppression (−) or stimulation (+) (%)	Macrophages suppression (−) or stimulation (+) (%)
CFW-mice AdCa 1×10^5	2	Nil	−49±3.9	+76±6.4	+69 ±5.8
	4	Nil	−21±1.9	+84±7.2	+77 ±6.3
	8	1±0.1	− 4±0.1	+32±2.3	+24 ±1.8
	16	7±0.5	27±1.8	−16±1.2	−29 ±2.1
	36	15±1.0	58±4.3	−53±4.8	−62 ±5.4
	Days after tumor removal*				
	2	—	52±4.7	−50±4.9	−60 ±5.9
	8	—	35±3.1	−48±4.2	−52 ±5.0
	16	—	3±0.4	−11±1.2	−19 ±1.7
	32	—	1±0.1	+ 5±0.4	+ 3 ±0.3
Rats-R3230 AdCa 1×10^5	4	Nil	−62±5.1	+52±5.1	+33 ±3.0
	8	Nil	−47±3.8	+62±5.1	+51 ±4.7
	16	Nil	−27±1.9	+80±7.6	+71 ±6.9
	22	1±0.1	17±1.4	+31±2.9	+23 ±1.8
	32	9±0.5	38±2.1	−40±3.1	−48 ±3.9
	42	19±1.7	62±4.2	−65±4.3	−71 ±5.4
	Days after tumor removal†				
	3	—	61±5.4	−64±5.1	−70 ±6.1
	9	—	44±4.2	−52±4.9	−61 ±5.8
	18	—	12±1.2	−23±1.9	−29 ±2.3
	38	—	5±0.5	−13±1.0	−17 ±1.7

The above data represent the average±S.D. of six separate experiments where 4 animals per group were used.
*The tumors were removed on day 36, after tumor cell inoculation.
†The tumors were removed on day 43, after tumor cell inoculation.

sion on quantitative correlation between serum and macrophage suppressing activities. The effect of tumor growth on other macrophage functions are under investigation.

6. *Reversibility of tumor-induced patterns by surgical removal of tumor mass*

Preceding experiments showed that serum suppressing and "blocking" activities, cell-

mediated cytotoxicity and macrophage-mediated suppression all varied with tumor size, the following experiments examine reversal of these patterns by removal of the tumor. The data in Table 4 showed that serum "blocking" activities increased, the initial serum and macrophage-stimulating activities changed to suppressing activities during tumor development. On the 36th and 42nd day after isograft, the tumors were removed surgically from both the AdCa-bearing mice and rats, respectively. After surgery, serum "blocking", and serum and macrophage suppressing activities decreased.

Therefore, these experiments demonstrated that surgical removal of the tumor reversed the patterns of serum and macrophage suppressing–stimulating activities. Whether the progressive growth of the tumor is the cause of the changes in the serum and macrophage activities, or the changes in these activities permitted and caused tumor progressive development must await further in progress studies.

DISCUSSION

Both CFW-AdCa in mice and R3230 AdCa in rats are hormone-regulated mammary adenocarcinomas. CFW-AdCa is also of moderate antigenicity [25] which yielded antigenic cell membrane fraction that was not found in the membrane fractions from normal mammary cells [26]. *In vivo* growth rate of this tumor was influenced by changes in the host immune reactivity, i.e., the serum from tumor-bearing animals enhanced, whereas the serum from immune animals retarded the growth of CFW-AdCa in syngeneic animals. Thus proliferation of CFW-AdCa in syngeneic unconditioned host was influenced by the host's immunological mechanisms.

Depending on the period after isograft, the serum from CFW-AdCa-bearing mice or R3230 AdCa-bearing rats caused stimulation and suppression of the response of normal syngeneic spleenocytes to PHA. This investigation supports the hypothesis, originally suggested by Prehn [13, 29, 30], of a dual effect of the immune response to a tumor transplant. Spleenocytes from normal mice were unable to stimulate target cell growth *in vitro* at low ratios of lymphoid cells to target cells (100:1). However, spleenocytes from CFW-mice or rats bearing the mammary adenocarcinoma for 5 days exhibited both stimulatory and inhibitory activities (unpublished preliminary observations). From the *in vivo* studies of Ilfeld *et al.* [31], Treves *et al.* [32], Carnaud *et al.* [33] and Small and Trainin [34], it would appear that stimulation and inhibition of tumor growth are mediated by different subpopulations of T-cells. However, the role of macrophages and B-cells in either phenomenon cannot be excluded. Fiddler [35, 36] found that cell-mediated immunostimulation depended on direct lymphoid cell-target cell contact. However, at least in the effect on the response of spleenocytes to PHA, our results indicated involvement of soluble factor(s). Cell-free supernatants from cultures of spleen cells [37] and peripheral blood leukocytes [38] exhibited both enhancing and cytotoxic effects on the DNA synthesis and division of target cells *in vitro*. Further, Shearer *et al.* [39, 40] have demonstrated humoral stimulation of tumor cell growth *in vitro* mediated by heterologous antibody.

Spleenocyte cytotoxic activities against syngeneic tumor cells, were detected early after inoculation of viable tumor cells and decreased sharply at later periods where the tumors were progressively growing. These results could be considered indicative, at least in the early period, of the presence of tumor-associated host response [9, 10], and in the latter period of the overwhelming mechanisms of the tumor. Although at low level, animals with greater tumor diameter maintained statistically significant ($P<0.001$) cell-mediated cytotoxic activity. Francois *et al.* [41] found that cell-mediated cytotoxicity disappeared entirely after 21 days, whereas Ladolfo *et al.* [42] reported that cytotoxic effector cells were present, although at low levels. The correlation between development of mammary adenocarcinoma and cytotoxicity is in agreement with reports in several tumor systems in which cytotoxic peak was detectable between 8 and 15 days after challenge [43–53]. The significance of the appearance of these peaks is not well understood. In some host-tumor systems cellular reactivity appears to be directly related to tumor size [46–49], it is quite possible that the peak of cellular activity is related to a general pattern of cytotoxic lymphocyte or other cellular activation.

In both CFW-AdCa and R3230 AdCa systems, other serum activities appear to be directly related to tumor size. For example at periods where the tumors became greater than 5 mm in diameter, the shift from serum

stimulating to suppressing activity was accompanied with appearance of serum "blocking" activity. Both, serum suppressing and "blocking" activities continued to increase until the animals expired. These two activities could be related to an increase of free or antibody-complexed membrane antigens shed by growing tumors [51–53]. It is possible that the suppressive activity may gradually become excessive, thus blocks the stimulating as well as the "arming" activity [54]. Further studies are in progress examining the possibility of the same compound acting as an immunosuppressive and as a "blocking" agent.

Macrophages may play a role in the control of tumor development. For example, a depression of a variety of macrophage functions resulted in an increased incidence of neoplasia and conversely, stimulation of various activities enhanced resistance to neoplastic disease [55]. Macrophages have been shown to influence blastogenesis in the mixed lymphocyte reactions (MLR). However, depending upon experimental conditions and animal species, blastogenesis may be enhanced or depressed by macrophages [56–58]. In the present studies, macrophages from either mice bearing CFW-AdCa or rats bearing R3230 AdCa suppressed response of normal syngeneic spleenocytes to PHA.

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Transient Bleomycin-Induced Thrombocytopenia. A Clinical Study

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Abstract—*The combination chemotherapy with vinblastine followed by a 5 day continuous infusion of bleomycin resulted in a transient decrease of the peripheral platelet count in all patients investigated. A significant bone marrow toxicity as a cause of thrombocytopenia could not be demonstrated. A detailed coagulation analysis, including fibrinogen turnover studies in one patient, confirmed the absence of intravascular coagulation as the cause for increased platelet consumption. Since no direct effects of bleomycin on blood platelets could be found, it is concluded that platelets are sequestrated in lung capillaries due to drug-induced early endothelial damage.*

INTRODUCTION

BLEOMYCIN is an antibiotic produced by a strain of streptomyces verticillus which has a broad spectrum of antiviral and antibacterial activity [1]. The drug was also shown to inhibit the growth of various transplanted animal tumours [2]. Bleomycin was found to be clinically effective in a variety of malignancies, predominantly squamous cell carcinomas [3]. As bleomycin is relatively little myelotoxic, it was incorporated into many chemotherapeutic regimens. In testicular cancers the combination of bleomycin with vinblastine is highly effective, resulting in total response rates of approximately 75% [4].

In our hospital, stage II testicular carcinomas were treated with a combination of vinblastine and bleomycin according to the schedule suggested by Samuels *et al.* [4]. Bleomycin is given over a 5-day period as a continuous intravenous infusion. Regularly after initiation of the therapy we observed a significant decrease of the peripheral platelet count. The present communication describes this phenomenon, and investigations to elucidate the pathogenesis of the bleomycin-induced thrombocytopenia are presented.

MATERIALS AND METHODS

Patients

Five patients were studied (age range: 22–38 yr) who had a semiorchidectomy with subsequent abdominal lymphadenectomy. In all patients the malignant disease was limited to the abdominal lymph nodes (stage II).

Treatment

Vinblastine was given on days 1 and 2 in a dose of 0.2 mg/kg/day followed by a continuous bleomycin infusion of 30 units/24 hr throughout days 2–6.

Laboratory tests

All tests were performed before and daily after initiation of the therapy. Red cells, leucocytes and blood platelets were determined using routine laboratory techniques. Plasma fibrinogen, prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT), reptilase time (RT) and factor VIII activity were measured according to standard procedures [5]. Factor XIII was estimated using the method of Bohn and Haupt [6]. Fibrin degradation products were studied in serum samples by the staphylococcal clumping test (SCT), the results being expressed as the exponents of the basis two [7].

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Fibrinogen turnover

In one patient the plasma half life ($t/2$) of ^{125}I-fibrinogen was determined during bleomycin therapy. Commercially available ^{125}I-fibrinogen (Radiochemical Centre, Amersham, U.K.) was used. Venous blood samples were taken every 12 hr after injection of the tracer and the fibrinogen $t/2$ was determined according to the method described by Bradley and Hickman [8]. The average mean $t/2$ had been previously established in 5 patients with acute leukaemia in complete remission and had been found to be 3.8 days (range: 3.0–4.9 days).

Bone marrow biopsy

In three patients the bone marrow was examined before and 7 days after initiation of the treatment. To compare the bone marrow composition before and after therapy unbiasedly, the bone marrow smears were coded.

In vitro *studies*

The blood platelets of two thrombocytopenic patients 5 days after initiation of the treatment were resuspended in normal, blood group identical plasma and incubated for 3 hr at 37°C. Similarly, platelets from two normal healthy donors were incubated in the plasmas of treated patients. The original platelet rich plasma served as a control. All platelet counts in the various samples were adjusted to $7 \times 10^4/mm^3$. Platelet counts of the samples were performed at various time intervals and finally a thrombelastogram was recorded after addition of calcium chloride to the plasmas.

Platelet rich plasma of two healthy control persons was incubated with different concentrations of bleomycin, ranging from 15 to 0.5 μg/ml, over a period of 1 hr, and platelet counts were performed at regular time intervals. Finally, thrombelastographic tracings were carried out after addition of calcium chloride to the plasmas. An equal volume of normal saline was added to the control samples.

RESULTS

Figure 1 shows the course of the platelet counts throughout the time of observation before and after therapy. Two to three days after initiation of the treatment a significant decrease of blood platelets was noted in all patients. The pattern of the leucocyte count during that period is given in Fig. 2. Leucopenia occurred 2 days after initiation of the therapy. No significant changes of the red cell count were observed. The plasma fibrinogen values before and during treatment are presented in Fig. 3. The

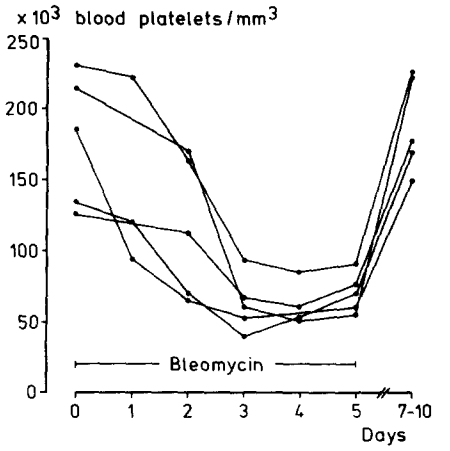

Fig. 1. Peripheral platelet counts in 5 patients during a 5 day continuous infusion of bleomycin.

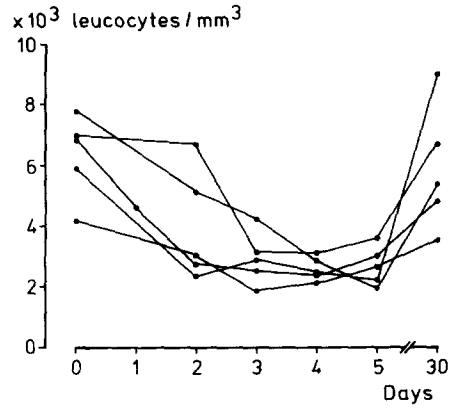

Fig. 2. Leucocyte count in 5 patients during a 5 day continuous infusion of bleomycin.

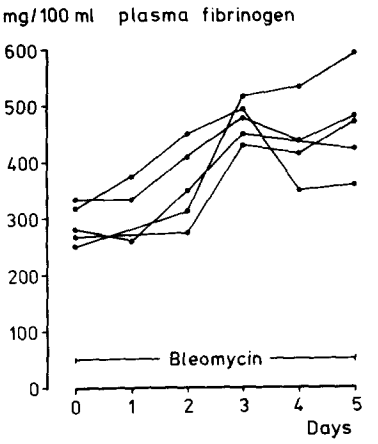

Fig. 3. Plasma fibrinogen levels in 5 patients during a 5 day continuous infusion of bleomycin.

fibrinogen levels increased concomitantly with the platelet decrease. Table 1 gives the mean values of the other coagulation data throughout the period of bleomycin treatment. There was no overall change of these parameters during treatment. Each laboratory value during treatment was subjected to a statistical analysis (analysis of variance) and compared to the pretreatment values. No significant changes could be elaborated in any of the parameters under investigation.

Table 1. Mean values of coagulation data of 5 patients during a 5 day continuous infusion of bleomycin (PT = Prothrombin time, PTT = Partial thromboplastin time, TT = Thrombin time, RT = Reptilase time, SCT = Staphylococcal clumping test)

	PT (%)	PTT (sec)	TT (sec)	RT (sec)	FVIII%	FXIII%	SCT (titre)
	mean	mean	mean	mean	mean	mean	mean
Day 0	96	45	18	16	100	120	4.8
Day 1	97	43	17	16	100	120	5.0
Day 2	73	42	18	15	100	114	4.6
Day 3	96	50	18	15	100	112	4.4
Day 4	97	42	18	17	100	106	4.2
Day 5	98	40	17	16	100	108	4.4
Normal range	75–100	35–52	17–21	14–18	80–100	80–120	3.0–6.0

The plasma half life of ^{125}I-fibrinogen in one patient during bleomycin therapy was found to be 4.1 days, indicating a normal survival of the clottable protein (normal range: $t/2 = 3.0$–4.9 days).

The bone marrow examinations in 3 patients before and after treatment revealed an obvious decrease of the cellularity in only one patient. A relative increase with a left shift of the granulopoesis and a decrease of the erythropoesis were seen in all patients. The morphology of the megakaryocytes appeared unaltered after therapy. By counting the number of megakaryocytes per slide under low power, a reduction was noted in one patient only.

Neither normal platelets resuspended in patients plasma nor platelets from bleomycin treated patients resuspended in normal plasma showed any numerical alterations throughout the incubation period. No gross evidence for a functional impairment of the platelets was observed in the thrombelastograms after recalcification of the platelet rich plasmas. Negative findings were also recorded when normal platelets were incubated with various amounts of bleomycin and compared to the corresponding control samples.

DISCUSSION

The main haematological feature in the 5 patients during bleomycin treatment was the significant decrease of circulating blood platelets 2–3 days after initiation of the continuous infusion. An identical phenomenon was seen in all patients subsequently subjected to this treatment. Since vinblastine is myelotoxic, thrombocytopenia could be attributed to the preceding injection of vinblastine. The simultaneous decrease of the white cells in our patients could suggest such a mechanism. However, although leukopenia is a frequent complication after vinblastine therapy, thrombocytopenia is a rare event [9]. Furthermore, if a platelet life span of 8–10 days [5] is taken as a basis for kinetic considerations, it is unlikely that the transient decrease of the peripheral platelet count by more than 50% within 2–3 days is a result of bone marrow damage. The bone marrow examinations carried out 7 days after initiation of the treatment support this assumption since no striking numerical or morphological alterations of the megakaryocytes could be seen.

If decreased platelet production is not the cause for the thrombocytopenia, an increased peripheral destruction has to be considered. Clinically, disseminated intravascular coagulation (DIC) is a major factor in the pathogenesis of increased platelet consumption. However, detailed coagulation studies in our patients did not reveal any laboratory evidence for DIC, on the contrary, a normal plasma fibrinogen turnover almost rules out this condition. Based on the *in vitro* studies it appears unlikely that bleomycin or any derivative thereof damages platelets directly in the circulation.

Among the toxicities of bleomycin, pulmonary lesions are clinically most important. The pathogenesis of pulmonary fibrosis, which according to a survey of 808 patients occurs in approximately 11%, is not fully understood [10]. After initiation of bleomycin treatment

in mice changes in the vascular endothelium of pulmonary capillaries have been demonstrated [11]. In contrast to previous investigations in humans, recently Burkhardt *et al.* [12] described a high incidence of intrapulmonary microthrombi after bleomycin treatment, suggesting that the activation of the clotting mechanism represents an initial step in the development of bleomycin-induced pulmonary toxicity. It has to be taken into account, however, that this study is based on autopsies, and antemortal pulmonary microemboli are a frequent observation in patients dying from chronic diseases [13]. The direct interrelation between bleomycin treatment and intrapulmonary microthrombi remains to be established.

From our data it can be concluded that bleomycin does not induce a hypercoagulable state, yet early endothelial damage in lung capillaries could lead to increased platelet sequestration at this site. Ultrastructural studies have clearly demonstrated that platelet aggregation can occur without fibrin formation at sites of endothelial damage, even before morphological alterations of the endothelium become visible [14]. It is conceivable that the peripheral platelet decrease after initiation of bleomycin treatment is an expression of such a mechanism. The concomitant increase of the plasma fibrinogen would favour this hypothesis, since tissue damage is frequently associated with an increase of the fibrinogen level [15]. Whether the observations described here are of any pathogenetic importance for the subsequent development of pulmonary fibrosis remains unknown. Experimental studies employing platelet aggregation inhibitors could possibly shed some light on the interaction of blood platelets, vascular endothelium and lung fibrosis after bleomycin treatment.

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Enrichment of *Helix pomatia* (HP) Lectin Binding Variant from the TA3St Mouse Ascites Tumor by Repeated Column Selection*

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Abstract—*TA3 is the ascites form of a mouse mammary carcinoma in strain A/Sn. It exists in two forms, the immunosensitive TA3St and the immunoresistant TA3Ha. The two lines also differ with regard to their ability to bind* Helix pomatia *A hemagglutinin (HP). We have attempted to select an immunoresistant subpopulation of TA3St by repeated enrichment of HP positive cells on HP-Sepharose columns. TA3St cells were passed through HP-Sepharose and the bound cells were eluted with the competitive hapten,* N*-acetyl-*D*-galactosamine* (D-*GalNAc*). *The eluted cells were reinoculated into syngeneic A/Sn mice, grown to ascites, harvested and again passed over a HP-Sepharose column in the same way. Following several selective cycles of this type, the* HP^+ *enriched cell fractions were further amplified by inoculating* 1–50 *cells into A/Sn mice. Subpopulations containing a high frequency of* HP^+ *cells were chosen for further column selections. We have successfully enriched* HP^+ *TA3St cells to* 95–100% *by this procedure. However, the selected variants had more weakly binding HP-receptors (low* K_{app} *and weak staining by HP-FITC) than the approximately* 5% HP^+ *cells in the original TA3St cell population or TA3Ha cells. They also differed from TA3Ha by remaining immunosensitive and by maintaining an unchanged H-2*^{a} *expression.*

INTRODUCTION

TA3St and TA3Ha are two serially transplanted ascites tumor lines, originating from the spontaneous mammary adenocarcinoma TA3 of strain A (H-2^a) origin [1–3]. The two sublines differ in H-2 antigen expression and in homotransplantability. TA3St grows only in syngeneic mice and has a high expression of H-2^a antigens (=immunosensitive) whereas the TA3Ha subline grows progressively across the H-2 barrier and sometimes even in rats and hamsters [1]. It has a low H-2 antigen expression (=immunoresistant) [4, 5].

Previously, the two lines have been compared with regard to a number of biological and biochemical characteristics, summarized in Table 1.

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Table 1. *Summary of some characteristics of TA3Ha and TA3St ascites tumor lines*

Characteristic	Tumor TA3Ha	TA3St
Growth in allogeneic mice	Yes	No
H-2^a antigen expression	Low	High
Susceptibility to humoral (anti H-2^a) cytotoxicity	No	Yes
Surface morphology	Smooth	Rough
Electrophoretic mobility	Fast	Slow
Con A agglutinability*	–	+
HP-receptors	Many	Few†
VG-receptors‡	Many	Few
Epiglycanin	Yes	No
Chromosomes	Near-diploid	Hypo-teraploid

Data from Friberg, [1, 2]; Friberg and Lilliehöök, [4]; Friberg and Hammarström, [9]; Codington, [8]; Codington *et al.* [6].

*Note that the number of surface receptors for Con A is the same for both tumor lines [9].

†Approximately 90% of the cells are HP receptor negative (see Table 2).

‡VG = *Vicia graminea* lectin.

The TA3Ha subline contains large amounts of a surface glycoprotein (epiglycanin) and large numbers of carbohydrate residues capable of combining with *Helix pomatia* A hemagglutinin (HP) and *Vicia graminea* lectin (VG). TA3St is low in epiglycanin, HP- and VG-receptors. Furthermore, the TA3Ha subline expresses very little H-2^a antigen, compared to the TA3St line. It has been postulated that the low expression of H-2^a on TA3Ha is related to the presence of the epiglycanin that would mask the H-2^a antigen [5]. This implies that the immunoresistance of this line is due to the same masking mechanism. It has been demonstrated that VG binds to the epiglycanin and that this substance is responsible for all, or almost all, of the binding to TA3Ha cells [6]. From considerations of the carbohydrate specificity of HP [7] the proposed chemical structure of the epiglycanin [8] as well as of the abundancy of epiglycanin and HP receptors on the TA3Ha cells it seems likely that HP similarly detects the epiglycanin on TA3Ha cells.

The present study was undertaken to gain more information about the immunoresistance phenomenon. We departed from our observation that TA3St cells were heterogenous with respect to HP-binding. While the majority ($\geqq 90\%$ of the cells) were HP negative, a small but variable fraction (5–10%) were HP positive by direct immunofluorescence with FITC-HP [9]. We have attempted to select an immunoresistant subpopulation of TA3St by repeated enrichment of HP positive cells on HP-Sepharose columns. This was done by passing TA3St cells through HP-Sepharose and eluting the bound cells with the competitive hapten, *N*-acetyl-D-galactosamine (D-GalNAc). The eluted cells were reinoculated into syngeneic A/Sn mice, grown to ascites, harvested and again passed over a HP-Sepharose column in the same way. Following several selective cycles of this type, the HP^+ enriched cell fractions were further amplified by inoculating 1–50 cells into A/Sn mice. Subpopulations containing a high frequency of HP^+ cells were chosen for further column selections. At intervals, selected and control cell populations were also tested for transplantability to foreign mouse strains and also for their ability to adsorb anti H-2^a antibodies.

We have successfuly enriched HP^+TA3St cells to 95–100% by this procedure. However, the selected variants had more weakly binding HP-receptors (low K_{app} and weak staining by HP-FITC) than the HP^+ cells in the original TA3St cell population or TA3Ha cells. They also differed from TA3Ha by remaining immunosensitive and by maintaining an unchanged H-2^a expression.

MATERIALS AND METHODS

Cell types

The history of TA3Ha and TA3St, two ascites sublines of a spontaneous mammary carcinoma that arose in a female strain A-mouse has been published together with the details of tumor maintenance [1, 3]. Table 1 summarizes some of their relevant properties.

TA3Ha/A.CA Cl. 3B AAA14-21, TA3Ha/A.CA Cl. 7 AAC10 and TA3Ha/A.CA Cl. 11 AAB16-24 are isoantegenic variant sublines of somatic cell hybrids, obtained by the fusion of $TA3HaB^{imp-}$ (an 8-AG resistant, HGPRT deficient, *in vitro* maintained subline of TA3Ha) with A.CA fibroblasts [3]. All hybrid lines were designated according to a system described by Friberg *et al.* [10]. The number following the initial three letters which designate the mouse genotype and individual cell line, represents the number of passages in A/Sn mice.

Originally, the hybrids were low malignant, but they threw off high malignant segregants following chromosome loss. These were reselected in syngeneic A × ACA F_1 hosts. Subsequently, H-2^f loss variants were selected by transfer to strain A mice and serial propagation in the ascites form [3, 10, 11].

Initially, the transplantability of the strain A compatible TA3Ha/ACA variants was restricted to syngeneic strain A mice and its cells had a normal H-2^a antigen expression [10, 11]. However, after 10–20 generations in A/Sn mice the sublines derived from clones 3B and 7 became allotransplantable and showed a decreased H-2^a expression in parallel [12].

The subline derived from clone 11 was only weakly allotransplantable and showed an almost normal H-2^a expression [12].

While these studies were in progress, an allotransplantable variant appeared spontaneously in a strain A mouse, inoculated with TA3St. This variant, designated as "TA3ST/NS" (non-specific) was also compared with the main lines with regard to HP-binding capacity.

Helix pomatia A hemagglutinin (HP) was purified by affinity chromatography on insolubilized hog blood group A+H substance [13].

It is a hexavalent carbohydrate binding protein with the following carbohydrate binding specificity (specificity range:

α-D-GalNAc > β-D-GalNAc ~ α-D-GNAc > β-D-GNAc > α-D-Gal) [7].

HP-receptor positive cells (HP^+ cells) were detected by direct membrane immunofluorescence. Fluoresceine isothiocyanate (FITC) conjugated HP was used. The F/P molar ratio was 7:14. $0.25–0.5 \times 10^6$ tumor cells were incubated with 0.1–15 μg FITC-HP in Tris buffered Hanks' solution (TH) containing 0.02% NaN_3 for 30 min at 4°C, washed with TH + NaN_3 and mounted in 50% glycerol . At least 200 cells were counted.

For binding studies HP was labelled with Na [^{125}I] (specific activity < 14 mCi/μg I) using the chloramine T procdeedure as described [14]. In the binding experiments a constant number of cells ($0.1–1.0 \times 10^6$) were incubated with increasing amounts of [^{125}I]-HP in a total volume of 2.7 ml phosphate buffered saline, pH 7.3 (PBS) containing 0.2% human serum albumin (HSA) and 0.02% NaN_3. The cells were kept in slow vertical rotation for 2 hr at 20°C. The binding data were treated according to Scatchard's derivation of the law of mass action [15]. The specificity of the binding and immunofluorescence was checked by the addition of D-GalNAc (positive control) and D-Glc (negative control).

Cell fractionation on HP-sepharose columns

Immunoadsorbent purified HP was conjugated to CNBr-activated Sepharose 6MB particles (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as earlier described [15]. Columns (0.9 × 15 cm) supplied with a 85 μm net, containing 3 ml swollen gel (1.5 mg HP/ml) were utilized. Tumor cells ($100–150 \times 10^6$) were applied to the columns and allowed to penetrate the gel. The cells were then incubated for 15 min in the gel. Non-adherent cells were washed off with 150–200 ml PBS containing 0.2% HSA. Bound cells were then eluted with 50 ml of PBS–HSA containing 3–5 mg D-GalNAc/ml. In some experiments the gel was then suspended in PBS–HSA + D-GalNAc and transferred to a test tube. The beads were allowed to settle and remaining cells in the supernatant were recovered. Cells recovered in this way amounted to about 5% of added cells. The specificity of the elution step was checked by the use of D-Glc instead of D-GalNAc. No cells (< 2%) were eluted with D-Glc.

Allograft tests

Undiluted ascitic fluid, 0.1 ml, containing approximately 10^7 ascites tumor cells, was inoculated intraperitoneally into syngeneic strain A mice, and into the following foreign genotypes: ABY (H-2^b), ASW (H-2^s), ACA (H-2^f), DBA/2 (H-2^d), CBA (H-2^k), C57Bl and C57leaden (H-2^b) and C3H (H-2^k). Mice were observed for tumor development during 2 months.

Absorption tests

An identical number of washed cells of each cell line at 7 serial dilutions ($400–6.3 \times 10^4$ cells) and controls, in triplicate, were incubated at 37°C for 45 min with A.CA anti H-2^a-antiserum (diluted 1:8 in RPMI medium containing 10% calf serum). After centrifuging down the cells, an aliquot of each supernatant solution was tested. Absorption was calculated from the cytotoxic action in the presence of guinea pig complement, compared to unabsorbed antisera, against normal A/Sn lymph node target cells, as previously described [3].

RESULTS

HP-receptors on different TA3-sublines

Table 2 summarizes the results of immunofluorescence and HP binding experiments. The percentage of HP positive cells and the average number of HP-receptors/cell was determined for different TA3-sublines. As shown earlier [9] all TA3Ha cells were strongly HP positive and contained large numbers of HP-receptors. Most TA3St cells were HP negative but there was a small, variable (4–13%) HP positive fraction. The average number of HP-receptors/cell was $0.2–0.6 \times 10^6$. When recalculated on the basis of the number of immunofluorescence positive cells (4–13%) *n*-values of $2–15 \times 10^6$ were obtained. These values are generally lower than those obtained for TA3Ha cells ($20–30 \times 10^6$).

We have also tested the strain A-compatible isoantigenic variants of three independently fused TA3Ha/ACA fibroblast hybrids (clones 3B, 7 and 11, for further nomenclature see footnote to Table 2). All three hybrid sublines were 90–100% HP positive and contained

Table 2. Binding of Helix pomatia *A hemagglutinin to different TA3-sublines-percentage of HP positive cells. Number of HP-receptors/cell* (n*) *and apparent association constant* (K_{app}) *for the interaction*

Tumor line		HP^+ cells (%)	n × 10^6	$K_{app} \times 10^{-8}$
TA3St 209 gen.		8	(8)†	0.6	2
214 gen.		13	(13)	0.3	2
217 gen.		4	(4)	0.6	1.5
219 gen.		5	(5)	0.2	2.5
224 gen.		6	(6)	0.2	2.5
TA3Ha 649 gen.		100	(100)	20	8
672 gen.		100	(100)	27	2.5
TA3Ha/A.CA‡ Cl 3B	AAA 14	100	(55)	13	2
	AAA21§	95–100	(65–85)	4.5–14	2–5
TA3Ha/A.CA Cl 7	AAC 10	100	(100)	3.5	11
TA3Ha/A.CA C 11	AAB 16	100	(45)	7	4
	AAB21¶	85–100	(25–65)	3–7	1.5–4
$TA3HaB^{imp-}$ *in vitro*		100	(60)	2	4
in vivo		100	(100)	10	2
TA3St/NS		7	(7)	0.1	2

*Number of receptors/cell calculated on total number of cells added.
†Strongly positive cells in parentheses.
‡TA3Ha/A.CA hybrids: clone 3B, 7 and 11 represent three independently established hybrids from the same original combination of parental cells [3].
AAA, AAB and AAC represent independently passaged sublines through strain A mice *in vivo*. The subsequent figure designates the number of serial passages *in vivo* in strain A mice.
§Four independent experiments on cells harvested after 8–19 days in A/Sn mice.
¶Four independent experiments on cells harvested after 6–15 days in A/Sn mice.

relatively large numbers of HP-receptors ($n = 3–14 \times 10^6$). The number of receptors and the percentage of strongly positive cells varied slightly on repeated tests of the same variant sublines. This variation did not appear to be related to the time of harvest of the ascites tumor cells 8–19 days after inoculation). Generally the variant sublines contained slightly fewer HP-receptors/cell than the TA3Ha line.

$TA3HaB^{imp-}$, an 8-AG resistant, HGPRT negative subline was tested both *in vivo*, in the ascites form and *in vitro*. There was an interesting difference in the number of HP-receptors. While both *in vivo* and *in vitro* cultivated cells were 100% HP positive the *in vivo* line contained about 5 times more receptors/cell than the *in vitro* line.

The spontaneously arising allotransplantable variant of TA3St, obtained in the course of this study and designated TA3St/NS, was also tested. As seen in Table 2, only a small fraction of the cells were HP positive and the number of receptors was low.

Origin of HP positive TA3St cells

The question arose whether HP positive cells of the TA3St subline were tumor or host cells. This was approached by a cytotoxic selection experiment (Table 3). TA3St cells were grown in A × ASW F_1 mice and in A/Sn mice. Ascites cells were harvested and exposed to an A anti ASW antiserum and rabbit complement. This can be expected to kill the host cells derived from the A × ASW F_1 but not the A/Sn mice. A/Sn and ASW lymphocytes were treated in the same way as controls. The antiserum killed the ASW lymphocytes and a significant fraction of the cells harvested from A × ASW F_1 mice inoculated with TA3St. Ascites cells harvested from A/Sn mice and control A/Sn lymphocytes were not killed. The percentage of HP positive cells was the same (5–6%) in the antiserum treated ascites cell populations harvested from both hosts, indicating that the HP positive cells were tumor cells. This is also in line with earlier studies, showing that peritoneal lymphoid cells from A/Sn and A × C3H mice were not stained by FITC-HP [9].

Purification of HP⁺ TA3St cells

HP-receptor containing TA3St cells were enriched by the selection procedure summarized in Table 4. Briefly, TA3St cells were applied to a column charged with HP, covalently bound to Sepharose 6MB particles. After washing with medium, bound cells were eluted with the competitive sugar

Table 3. HP positive cells in A anti-ASW antiserum + complement treated TA3St ascites cell populations grown in A × ASW F_1 and A/Sn mice respectively

Cells	No. treatment living cells (%)	Antiserum + C treatment living cells (%)	HP⁺ (%)
TA3St in A × ASW F_1	97	67	6
TA3St in A/Sn	93	87	5
ASW-lymphocytes	87	2	
A/Sn-lymphocytes	60	50	

Table 4. Enrichment of HP⁺ TA3St cells by repeated HP-sepharose column fractionation and cell cloning

Selection procedure and selection step No.		Before selection	HP⁺ cells (%) HP-Sepharose column Passed cells	Eluted cells*	Clonal harvest
HP-Seph. column No	1	8	0	9	—
	2	8	3	12	—
	3	10	2	20	—
	4	29(8)†	4(4)	55(13)	—
	5	15	3	34	—
	6	15	3	39	—
	7	7	0	14	—
Cloning in A/Sn mice‡		14	–	—	25
HP-Seph. column No. 8 (×2)		25	NT§	88	—
Cloning in A/Sn mice‡		88	–	—	40(8)
HP-Seph. column No.	9	40(8)	NT	89(4)	—
	10	37(1)	NT	51(1)	—
	11	27(2)	NT	76(2)	—
	12(×2)	66(6)	11(1)	95(3)	—
	13	70(3)	20(1)	63(7)	—
	14	47(11)	NT	NT	—
	15	18(4)	NT	51(4)	—
	16	54(6)	NT	80(20)	—
	17(×2)	67(3)	26(0)	90(0)	—
	18	86(0)	NT	93(0)	—
	19	92(2)	NT	100(2)	—
HP-Seph. column No.	20	45(7)¶	NT	58(6)	—
	21	77(5)	NT	67(3)	—
	22	36(2)	NT	90(2)	—
	23	81(6)	NT	94(0)	—
	24	100(3)	NT	100(3)	—
	25	100(3)	NT	98(1)	—

*After each column fractionation the D-GalNAc eluted cells were passaged in A/Sn mice.

†Denotes strongly positive cells.

‡A statistical average of one cell was inoculated into A/Sn mice i.p. and the resulting ascites tumor was harvested.

§NT not tested.

¶These cells were from HP-Sepharose column No. 19 (D-GalNAc eluted fraction). The cells were passaged in A/Sn mice and frozen. Five months later the cells were inoculated in A/Sn mice and the resulting ascites tumor was used.

hapten D-GalNAc. Eluted cells were passaged i.p. in A/Sn mice and the fractionation procedure was repeated. After 7 consecutive column fractionations and mouse passages a cloning experiment was performed. A statistical average of 1 D-GalNAc eluted cell per mouse, obtained from HP-column 7, was injected into a series of A/Sn mice. An ascites tumor grew out in one out of 25 inoculated mice only (25% HP^+ cells). These cells were fractionated on two HP-Sepharose columns (88% HP^+ cells) and subjected to a second cloning. Growth was obtained in one out of 5 mice (40% HP^+ cells). These cells were used for further column selection experiments (Table 4). Table 4 shows that the percentage of HP positive cells increased gradually during the selection procedure, reaching 90–100% HP^+ cells after 17 column fractionations and mouse passages. There are three points of interest. (1) The efficiency of each fractionation procedure was relatively low. Only a 2–3 fold enrichment of HP^+ cells was obtained in the sugar eluted fraction at its best. On the other hand, HP^+ cells were efficiently depleted from the cell population, provided the columns were not overloaded. (2) Only weakly stained HP^+ cells were enriched by the column selection procedure. Somewhat surprisingly the strongly stained cells were actually lost. Calculation of the recovery of the HP-marker (positive and strongly positive) in 5 experiments showed that 70–80% of the HP^+ cells were recovered in the various fractions passed, D-GalNAc-eluted and mechanically eluted in presence of D-GalNAc). In contrast only 25–35% of the strongly positive cells were recovered. Inspection of the beads after mechanical elution showed that they were free of cells. (3) Passage of the eluted cells in A/Sn mice (6–13 days) decreased the percentage of HP^+ cells, as a rule.

Properties of HP^+ TA3St cells

Figure 1 shows the binding of radiolabelled HP to untreated and to HP^+ enriched TA3St cells at different stages of the selection procedure. Biphasic binding curves were obtained with the HP^+ enriched TA3St cells, reflecting the presence of strong and weak HP-binding receptors. Weak HP-binding receptors could not be demonstrated in the original TA3St cell population. After 19 consecutive HP-Sepharose selections when the cells were 100% HP positive (see Table 4), the number of strong and weak HP-binding receptors per cell was 1.2×10^6 and 3.2×10^6, respectively. The K_{app} for the interaction between HP and strong binding receptors on the HP^+ enriched TA3St cells was similar to the value for the interaction between HP and the original TA3St cells.

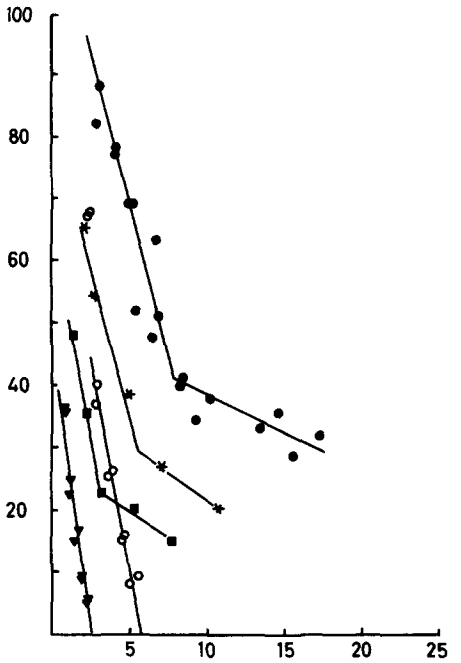

Fig. 1. Binding of [^{125}I]-labelled HP to TA3St cells of generation 224 (▼——▼) *and* 217 (○——○), *respectively and to* D-*GalNAc eluted TA3St cells after* 11 (■——■). 13 (*——*) *and* 19 (●——●) *fractionations on HP-Sepharose columns, respectively see Table* 4). *Abscissa:* $\frac{r}{[H]} \times 10^{-12}$. *Ordinate:* $r \times 10^{-5}$.

The K_{app} for the interaction between HP and weak binding receptors was approximately one tenfold lower. It may therefore be concluded that the fractionation procedure has selected a new population of TA3St cells characterized by a relatively large number of weak HP-binding receptors. These cells could not be demonstrated in the original TA3St tumor either by immunofluorescence or by HP-binding (Table 2 and Fig. 1).

Repeated passage of HP^+TA3St cells in A/Sn mice without intermittent column fractionation led to the gradual loss of cells with weak HP-binding receptors (Table 5). After 12 passages the frequency of HP^+ cells had decreased to 23%. This cell population gave a nearly monophasic binding curve, showing the presence of strong binding sites and only few weak binding sites. It may therefore be concluded that the TA3St cell population obtained by the column selection procedure described above (Table 4) has reverted back to a cell population with

Table 5. Loss of HP^+ cells by repeated passage of HP^+ TA3St cells in A/Sn mice

Passage No.	HP^+ cells (%)
0	100* (2)†
1	94 (2)
2	96 (10)
3	93 (3)
4	91 (6)
5	100 (2)
6	92 (3)
7	48 (6)
8	NT‡
9	36 (22)
10	41 (19)
11	25 (5)
12	23 (2)

*TA3St cells obtained after 19 successive HP-column selections, with intermittent mouse passage (see Table 4).
†Denotes strongly positive cells.
‡NT not tested.

HP-binding characteristics similar to those of the original TA3St cells. This reversion was characterized by loss of cells with weak HP-binding receptors. Cells with strong HP-binding receptors occurred in about the same proportion in both the column selected —and reverted cell populations and in the original TA3St population.

Allograft tests

In view of the fact that the highly HP-reactive TA3Ha cell population grows apparently without limitations in allogeneic mice, we have tested various HP^+ enriched TA3St cell populations for their ability to grow in allogeneic mice. The cells were tested after the following selection steps: HP-Sepharose column No. 6 + mouse passage ($HP^+ = 7\%$), column No. 13 + mouse passage ($HP^+ = 47\%$), column No. 16 + mouse passage ($HP^+ = 67\%$), column No. 17 + mouse passage ($HP^+ = 86\%$), column No. 20 + mouse passage ($HP^+ = 77\%$), column No. 22 + mouse passage ($HP^+ = 81\%$), column No. 23 + mouse passage ($HP^+ = 100\%$), column No. 24 + mouse passage ($HP^+ = 100\%$) and HP-Sepharose column No. 19 + 5 mouse passages ($HP^+ = 100\%$) (c.f. Tables 4 and 5). Altogether we have inoculated 8 A/Sn, 10 ABY, 8 ACA, 10 ASW, 9 DBA/2, 6 C57leaden, 6 C3H, 10 C57Bl, 8 CBA and 10 C57Bl × DBA/2 F_1 hybrid mice. All strain A mice died with progressively growing tumors. Occasional temporary growth was seen in a few ABY, DBA/2 and C3H mice but these invariably regressed. No growth was seen in any of the other allogeneic hosts and none of them died with tumors, under an observation period of more than 2 months.

Absorption tests

HP^+ enriched TA3/Stockholm cell populations were also tested for their ability to bind anti-H-2^a antibodies, in comparison with TA3St och TA3Ha cells, as described previously [3].

Altogether 4 absorption experiments were carried out against an ACA antiA serum, tested against normal A lymphocytes as targets. There was no significant difference in the absorbing capacity of TA3St and the HP^+ enriched population.

DISCUSSION

These data show that it was possible to select, from TA3St cell populations, HP-binding cell fractions by a series of HP-column binding and elution sequences. However, the HP-binding properties of the selcted HP^+ TA3St line differed considerably from the TA3Ha line. The findings are schematically summarized in Table 6.

Biologically, the selected variants did not correspond to TA3Ha because they failed to grow in foreign strains of mice after homotransplantation and remained unchanged with regard to the absorbing capacity for anti-H-2^a antibodies. This suggests that the membrane changes responsible for the high HP binding do not necessarily involve the masking of H-2^a antigenic sites. As a subsequent step, we

Table 6. Schematic summary of HP-binding, allotransplantability and H-2^a expression

Cell line	HP-receptors strong	HP-receptors weak	Allotransplantability	H-2^a expression
TA3St	2–10%	—	–	high
HP^+TA3St	2–10%	>90%	–	high
TA3Ha	100%	—	+	low

intend to select allotransplantable TA3St sublines by forced passage through foreign strains, to see whether this biological selection leads to the appearance of epiglycanin positive variants with high HP-binding capacity and reduced H-2^a antigen expression.

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Effect of Human and Mouse Interferon and of Polyriboinosinic Acid·Polyribocytidylic Acid on the Growth of Human Fibrosarcoma and Melanoma Tumors in Nude Mice

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Abstract—*Human leukocyte interferon, human fibroblast interferon, mouse interferon and an interferon inducer, polyriboinosinic acid·polyribocytidylic acid (poly(I)·poly(C)), were examined for their effect on the growth of two human tumor cell lines in athymic nude mice. The human tumor cell lines were derived from a fibrosarcoma (designated HT-1080) and a nonmelanotic melanoma (designated A-375). Repeated doses of* 5 mg/kg *of poly(I)·poly(C), administered every other day starting* 1 day *after tumor cell inoculation, caused a significant inhibition of tumor growth. On the contrary, human (leukocyte or fibroblast) interferon and mouse interferon, at doses up to* 5×10^6 units/kg, *did not affect the growth of either of the two human tumor cell lines tested.*

INTRODUCTION

CONGENITALLY athymic (nude) mice accept for their lifetime skin grafts from mammals (cat, human), birds (chicken), reptiles (lizards) and amphibians (frogs) [1, 2]. They also support the growth of a variety of human tumor cells. Both primary human tumors, e.g. malignant melanoma [3] and lung carcinoma [4] and permanent cell lines derived from human tumors, e.g. melanoma [5–7], HeLa [8], mammary carcinoma [9], colon carcinoma [10] and neuroblastoma [11] have been transplanted successfully to nude mice. Even after long-term serial transplantation in nude mice, these malignant tumors retain their original cytological and histological appearance [12], although Aubert *et al.* [5] noted ultrastructural and biochemical changes in human malignant melanoma cell lines which had been transplanted into nude mice. The human tumor-nude mouse system has been advocated as a suitable model for *in vivo* screening of sensitivity of human tumors to chemotherapeutic agents [13].

We have been able to maintain in nude mice the growth of two established human tumor cell lines, a fibrosarcoma (designated HT-1080) and a nonmelanotic melanoma (designated A-375). These tumor systems were chosen to evaluate the antitumor potentials of interferon and poly(I)·poly(C). Both human interferon and mouse interferon were examined for antitumor activity. We reasoned that if interferon exerted a direct cytotoxic (or cytostatic) effect on the tumor cells, human interferon but not mouse interferon would prove to be effective. If, however, the antitumor effect of interferon were mediated by the host cells (e.g. lymphocytes or macrophages), one might expect mouse interferon but not human interferon to be effective. *In vitro* and *in vivo* interferon generates several biological responses some of which may seem compatible with a direct

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inhibitory effect on tumor cell growth while others point to a host-mediated rejection of the tumor cells. For example, interferon inhibits the multiplication of leukemic cells under steady-state conditions [14]. It enhances the expression of cell surface antigens [15], including the expression of histocompatibility antigens on lymphoid cells [16, 17]. Furthermore, interferon has been shown to increase the specific cytotoxicity of sensitized lymphocytes for allogeneic target tumor cells [18] and to stimulate the phagocytic activity of peritoneal macrophages [19, 20].

By virtue of its interferon inducing capacity and several other activities, in particular a direct inhibitory effect on macromolecule (DNA, RNA and protein) synthesis [21–23], poly(I)·poly(C) might also be expected to exhibit an antitumor action in the human tumor-nude mouse system. Poly(I)·poly(C) has recently been found to suppress the growth of mouse L-929 tumor cells in nude mice [24]. This antitumor effect of poly(I)·poly(C) may have resulted from a direct cytotoxic action of the double-stranded RNA on the tumor cells. Poly(I)·poly(C) has indeed been shown to selectively block macromolecule synthesis of tumor cells [25].

MATERIALS AND METHODS

Mice

Athymic nude (*nu/nu*) mice of NMRI background were bred according to breeding scheme IV of Giovanella and Stehlin [26]. They were kept in stainless steel cages, in conventional conditions, at an ambiant temperature of 25°C and a relative humidity of 80%. Other characteristics of our nude mouse strain have been dealt with previously [24].

Interferon preparations

Human leukocyte interferon was kindly provided by Dr. K. Cantell (Central Public Health Laboratories, Helsinki, Finland). The human leukocyte interferon had a specific activity of 10^6 U per mg protein.

Human fibroblast interferon was prepared essentially as described by Billiau *et al.* [27], except that the final medium contained 6 mM Hepes (*N*-2-hydroxyethyl piperazine), 13 mM tricine (*N*-Tris–hydroxymethyl-methyl glycine) and 1% (v/v) pasteurized human plasma protein fraction (Belgian Red Cross). Two human fibroblast interferon preparations were employed. The first preparation had a specific activity of 2×10^5 U/mg protein and was used in these animals which were treated with interferon at 5×10^5 u/kg (10^4 U per mouse). The second preparation, which had been purified by adsorption chromatography on controlled pore glass [28], had a specific activity of 2×10^6 U/mg protein and was used in these animals which were treated with interferon at 5×10^6 U/kg (10^5 U per mouse).

Mouse interferon was induced in L-929 cells by Newcastle disease virus. It was prepared according to a modification of the procedure described by Knight [29]. The specific activity of the mouse interferon preparation amounted to 6×10^5 U/mg protein.

Interferon titers

For human leukocyte interferon, the titers are expressed in terms of the Human Interferon Research Standard B (MRC 69/19). For human fibroblast interferon such standardization is not applicable, since human fibroblast and leukocyte interferons show different dose–response curves [30, 31]. Therefore, human fibroblast interferon titers are expressed in laboratory reference units. Mouse interferon titers are expressed in terms of the Mouse Interferon Reference Standard G 002-904-511 (NIH).

Poly(I)·poly(C)

Poly(I)·poly(C) was prepared by mixing equal volumes of the homopolymer solutions of poly(I) and poly(C), both purchased from P-L Biochemicals (Milwaukee, Wisconsin) and dissolved in Dulbecco's PBS (phosphate buffered saline) at 1 mg/ml. Aliquots of the poly(I)·poly(C) stock solution were stored at −20°C and thawed prior to use.

Tumor cell lines

The fibrosarcoma HT-1080 cell line was established by Rasheed *et al.* [32] from the fibrosarcoma of a 35 yr old Caucasian man who died without having received chemotherapy or radiotherapy.

The melanoma A-375 cell line was provided by Dr. P. T. Allen (National Cancer Institute, Fort Detrick, Frederick, U.S.A.).

The HT-1080 and A-375 cell lines were maintained in polystyrene culture bottles in Eagle's minimal essential medium supplemented with non-essential aminoacids, tricine

and 10% heat-inactivated (90 min at 56°C) fetal calf serum. The cells were subcultured weekly by trypsinization at a 1–10 split ratio.

Histological and ultrastructural examination

For histological studies the tumors were fixed in formol, embedded in paraffin and stained with erythrosin-hematoxylin (or Gordon–Sweets to visualize reticulin). For ultrastructural studies, tumor specimens were fixed in glutaraldehyde, postfixed with osmium tetroxide, stained with uranyl acetate, dehydrated and embedded in araldide. Thin sections were cut with an OMU 2 Reichert ultramicrotome, stained with lead hydroxide and examined by a Zeiss 95 electron microscope.

Tumor cell inoculation

The antitumor activity of interferon and poly(I)·poly(C) was assessed with fibrosarcoma HT-1080 and melanoma A-375 tumors which had been passed previously onto nude mice. To this end, the tumors were harvested from the mice and after trypsinization, the tumor cells were resuspended in serum-free Eagle's minimal essential medium. The tumor cells were then injected subcutaneously with a 22 G 1 1/4 needle in the dorsum of 22 day old *nu/nu* mice. The HT-1080 cells were injected at 3×10^6 cells/0.1 ml/mouse whereas the A-375 cells were injected at 1.2×10^6 cells/0.1 ml/mouse.

Interferon treatment

The interferon preparations (mouse interferon as well as human fibroblast or leukocyte interferon) were injected intraperitoneally to the nude mice at either of the following dosage regimens: (i) 10^4 U per mouse (*ca.* 5×10^5 U/kg) on alternate days from the first till the 23rd day after tumor cell inoculation, or (ii) 10^5 U per mouse (*ca.* 5×10^6 U/kg) on alternate days from the first till the 9th day after tumor cell inoculation. The interferon doses employed in our studies are comparable to those which are generally feasible in man and exceed those (3×10^6 interferon units per person) which are now being administered to patients with osteogenic sarcoma [33, 34].

Poly(I)·poly(C) treatment

Poly(I)·poly(C) was injected intraperitoneally at 100 μg per mouse (*ca.* 5 mg/kg) on alternate days from the first till the 9th or 23rd day after tumor cell inoculation. The latter treatment regimen has been shown previously [24] not to impair the normal life span of nude mice and may be considered equivalent to the poly(I)·poly(C) dosage that has been employed in preliminary studies in human tumor patients [35, 36].

Measurements of tumor size

Length and breadth of the tumors were measured once weekly with slide calipers. The third dimension of the tumors could not be determined accurately. Tumor size is presented as the square root of the tumor area (calculated in mm^2).

Statistical analysis

Differences in tumor size between two groups at a given time were assessed by Student's *t*-test.

RESULTS

Effect of interferon and poly(I)·poly(C) on fibrosarcoma HT-1080 tumor growth in nude mice

Tumors developed at the inoculation site of the cells, grew as single encapsulated masses with spherical, ellipsoid or rectangular form and displayed the same growth pattern in male and female mice. Spontaneous regression was not observed. Tumor development did not hamper the animal's activity or hasten its death. Tumor growth was local. There was no evidence of macroscopic metastases.

On histological examination the tumor appeared to be composed of poorly differentiated cells with large nuclei and prominent nucleoli. Silver staining revealed the presence of reticulin fibers. On electron microscopic observation, collagen fibrils were found outside the cells. The cytoplasm of the cells contained filaments which may be identified as protocollagen fibrils.

Neither human (leukocyte or fibroblast) interferon nor mouse interferon, administered intraperitoneally at 10^4 U per mouse on alternate days, starting one day after tumor cell inoculation, had an appreciable effect on tumor growth (Fig. 1). Under the same conditions, poly(I)·poly(C), administered at 100 μg per mouse, caused a marked inhibition of tumor development. The average tumor sizes ($\sqrt{mm^2}$) recorded at day 43, that is 21 days after tumor cell inoculation, were 19.0 for group I (human leukocyte interferon), 17.0 for group II (human fibroblast interferon), 16.2 for group III (mouse

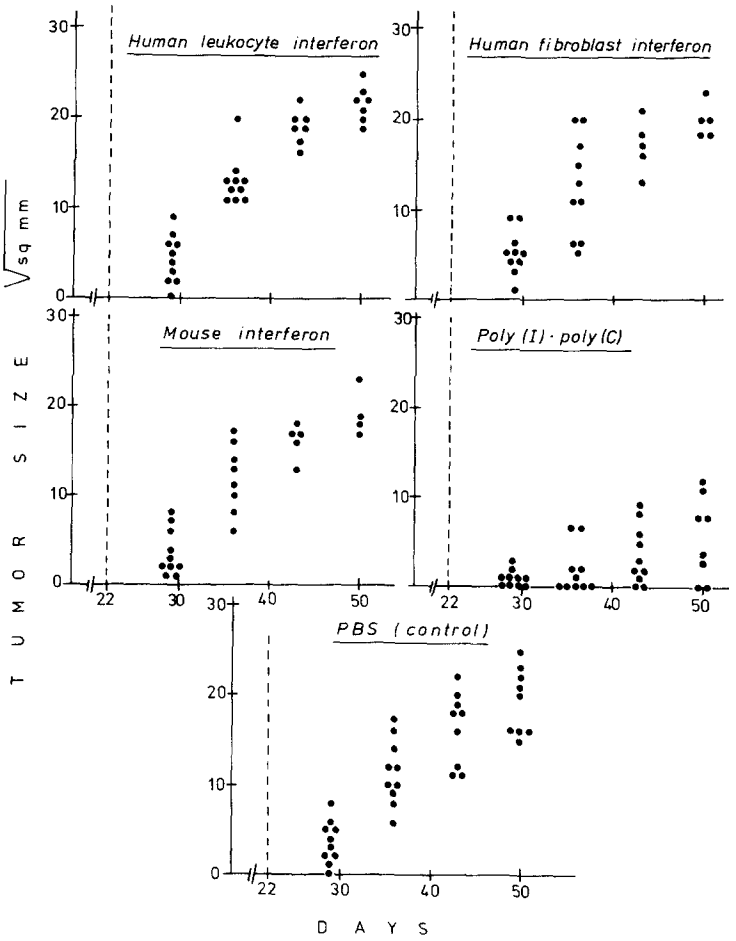

Fig. 1. Effect of human leukocyte interferon, human fibroblast interferon, mouse interferon and poly(I)·poly(C) on fibrosarcoma HT-1080 tumor growth in nu/nu *mice. Human leukocyte interferon* (10^4 U/*mouse*), *human fibroblast interferon* (10^4 U/*mouse*), *mouse interferon* (10^4 U/*mouse*) *and poly(I)·poly(C)* (100 μg *mouse*) *were injected intraperitoneally at* 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 *and* 23 days *after tumor cell inoculation. Tumor cells* (3×10^6 *cells/mouse*) *were inoculated subcutaneously on the* 22nd day *of life. Dots represent tumor sizes of individual mice at different days of age.*

interferon), 3.6 for group IV [poly(I)·poly(C)] and 16.3 for group V (PBS control). While differences in tumor size between the control (group V) and groups I, II and III were not significant, tumor sizes of mice which had been treated with poly(I)·poly(C) (group IV) significantly differed from tumor sizes of control mice (group V) ($P<0.001$).

At the dosage regimen at which poly(I)·poly(C) was employed, there was no evidence of a deleterious effect on the general health (body weight) of the animals. When the poly(I)·poly(C) injections were stopped, there was no burst of tumor growth. The tumors grown in poly(I)·poly(C)-treated mice and the tumors grown in untreated mice did not reveal any histological or ultrastructural differences.

As neither human interferon nor mouse interferon exerted an inhibitory effect on HT-1080 tumor growth when injected at 10^4 U per mouse, additional experiments were performed in which both mouse and human interferon were administered at 10^5 U per mouse. This treatment was initiated one day after tumor cell inoculation and continued every other day until the ninth day after tumor cell inoculation, when it became apparent that even at 10^5 U per mouse human interferon as well as mouse interferon failed to affect HT-1080 tumor growth (Fig. 2). The average tumor sizes ($\sqrt{mm^2}$) recorded at day 34, that is 12 days after tumor cell inoculation, were 11.0 for the group of mice treated with human fibroblast interferon, 11.1 for the group of mice treated with mouse interferon and 10.7 for the

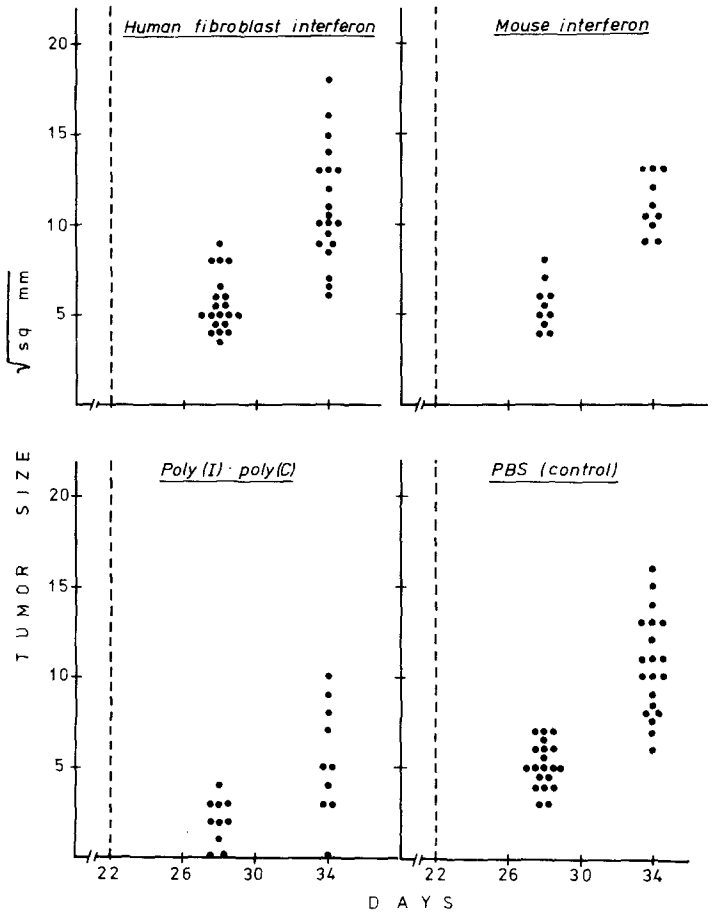

Fig. 2. Effect of short-term treatment with human fibroblast interferon, mouse interferon and poly(I)·poly(C) on fibrosarcoma HT-1080 tumor growth in nu/nu *mice. Human fibroblast interferon* (10^5 U/*mouse*), *mouse interferon* (10^5 U/*mouse*) *and poly(I)·poly(C)* (100 μg/*mouse*) *were injected intraperitoneally at* 1, 3, 5, 7 *and* 9 days *after tumor cell inoculation. Tumor cells* (3×10^6 *cells/mouse*) *were inoculated subcutaneously on the* 22nd day *of life. Dots represent tumor sizes of individual mice at different days of age.*

control (PBS) group. Five consecutive doses of 100 μg of poly(I)·poly(C) administered at 1, 3, 5, 7 and 9 days after tumor cell inoculation sufficed to reduce the average tumor size (measured at the 12th day after tumor cell inoculation) from 10.7 (control) to 5.4 ($P<0.001$) (Fig. 2).

As the lack of antitumor activity of human interferon might have been attributed to an abnormally rapid clearance (or inactivation) or human interferon in the bloodstream of nude mice, interferon titers were measured in the blood of HT-1080 tumor-bearing mice which had received multiple intraperitoneal injections of either human (fibroblast) interferon or mouse interferon (10^5 units/mouse) or poly(I)·poly(C) (100 μg/mouse) (regimen as explained in the legend to Fig. 2). Comparable serum interferon titers, amounting to approximately 1000 units/ml, were obtained within 2–8 hr following the fifth injection of poly(I)·poly(C) or of human and mouse interferon (data not shown), suggesting that the inability of human interferon to inhibit HT-1080 tumor growth in the nude mouse could not be attributed to an unusually rapid disappearance of human interferon from the blood. Injection of mouse interferon to nude mice resulted in the appearance of serum interferon activity with the species specificity of mouse interferon, and injection of human interferon gave rise to serum interferon activity with the species specificity of human interferon. It is noteworthy that upon injection of poly(I)·poly(C) only mouse interferon was detected in the bloodstream; human interferon might have been found if the transplanted HT-1080 tumor cells participated in the production of interferon.

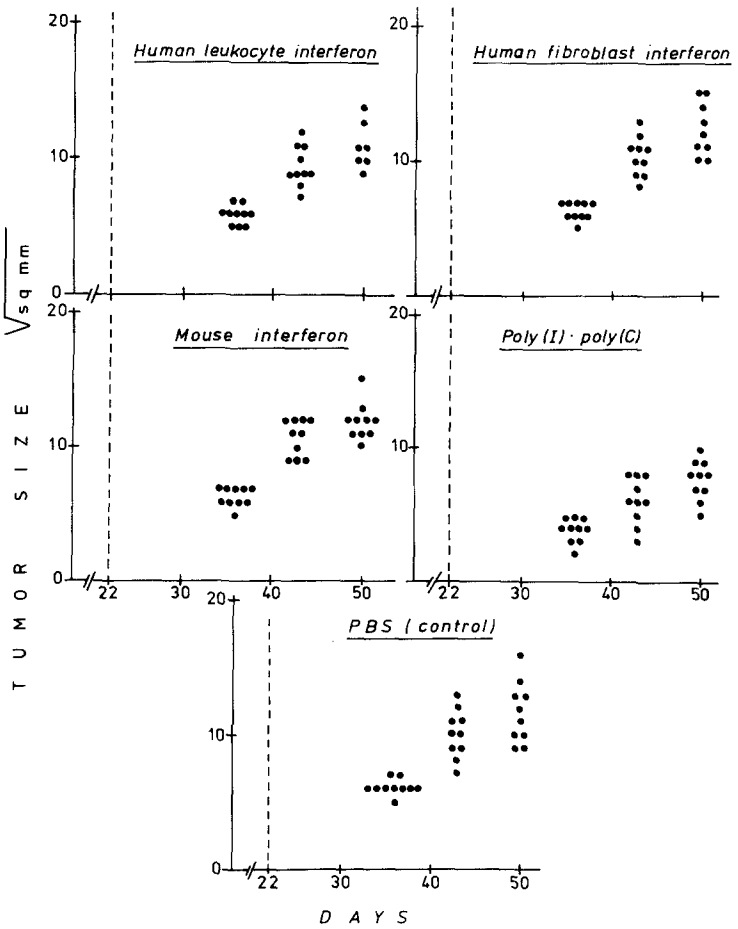

Fig. 3. Effect of human leukocyte interferon, human fibroblast interferon, mouse interferon and poly(I)·poly(C) on melanoma A-375 tumor growth in nu/nu *mice. Human leukocyte interferon* (10^4 U/*mouse*), *human fibroblast interferon* (10^4 U/*mouse*), *mouse interferon* (10^4 U/*mouse*) *and poly(I)·poly(C)* (100 μg/*mouse*) *were injected intraperitoneally at* 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 *and* 23 days *after tumor cell inoculation. Tumor cells* (1.2×10^6 *cells/mouse*) *were inoculated subcutaneously on the* 22nd day *of life. Dots represent tumor sizes of individual mice at different days of age.*

Effect of interferon and poly(I)·poly(C) on melanoma A-375 tumor growth in nude mice

The growth patterns demonstrated by the melanoma A-375 tumors was essentially the same as that of the fibrosarcoma HT-1080 tumors, except that tumors grew at a slower pace. Also, melanoma tumors assumed a predominantly spherical form.

Histological examination of the tumor revealed the presence of pleomorphic cells, numerous mitotic figures, but no melanin. Premelanosomes were detected upon electron microscopic observation. Melanization was incomplete as could be expected from an aggressive melanoma.

One day after they had been inoculated with the melanoma tumor cells, the mice were submitted to the same interferon treatment as employed for the fibrosarcoma HT-1080 cell-bearing mice. As shown in Fig. 3, both human interferon, whether leukocyte or fibroblast, and mouse interferon failed to affect tumor growth. Poly(I)·poly(C), however, brought about a partial reduction of tumor size. The average tumor sizes ($\sqrt{\text{mm}^2}$) recorded at day 43, that is 21 days after tumor cell inoculation, were 9.5 for group I (human leukocyte interferon), 10.4 for group II (human fibroblast interferon), 10.7 for group III (mouse interferon), 6.1 for group IV [poly(I)·poly(C)] and 10.0 for group V (PBS control). The tumor sizes of groups I, II and III did not significantly differ from that of the control (group V). However, differences in tumor size between the control group and group IV (which had been treated with poly(I)·poly(C)) were significant at $P<0.001$.

DISCUSSION

In mice, poly(I)·poly(C) has been shown to inhibit the growth of a variety of transplanted murine tumors, e.g. L-1210 leukemia [37], reticulum cell sarcoma [38], malignant melanoma [39] and mammary carcinoma [40]. These inhibitory effects have recently been extended to L-929 tumor cell growth in nude mice [24] and are further extended, in the present report, to two human tumor cell lines transplanted to nude mice. The two cell lines tested were a fibrosarcoma (designated HT-1080) and a nonmelanotic melanoma (designated A-375). Upon subcutaneous inoculation of 1 or 3×10^6 tumor cells, single, well-defined tumor masses developed at the sites of inoculation. Repeated doses of 100 μg of poly(I)·poly(C) (5 mg/kg), administered intraperitoneally on alternate days starting 1 day after tumor cell inoculation, effectively inhibited tumor growth. When tested under the same conditions, no inhibition of tumor growth was obtained with poly(I)·poly(s^2C), a thioketo analogue of poly(I)·poly(C) (41), kindly provided by Dr. K. Reuss (E. Merck, Darmstadt) (data not shown). Human interferon and mouse interferon, administered at 10^4 or 10^5 per mouse (5×10^5 or 5×10^6 U/kg), also failed to affect tumor growth.

Yet, mouse interferon preparations have been reported to inhibit the growth of several murine transplantable tumors and to increase the survival of the tumor-inoculated mice [42–45]. These mouse interferon preparations were particularly effective when intimate contact between tumor cells and interferon occurred, i.e. in mice which received both the tumor cells and the interferon preparations by the intraperitoneal route [42, 44]. However, interferon has also been shown to inhibit the growth of a solid malignant tumor (Lewis lung tumor) and to prevent the development of pulmonary metastases in experiments in which the tumor cells were inoculated subcutaneously and interferon was administered by the intravenous route [43]. In these conditions as well as in ours, no direct contact between tumor cells and interferon was achieved. Whereas interferon blocked tumor cell proliferation in the one case [43], it failed to do so in the other (see Figs. 1–3).

The antitumor activity demonstrated by poly(I)·poly(C) in the nude mouse may be ascribed to a direct cytotoxic action of the drug on tumor cell metabolism. Both HT-1080 fibrosarcoma cells and A-375 melanoma cells proved particularly susceptible to the cytostatic effects of poly(I)·poly(C) [as monitored by (^{3}H-methyl) thymidine incorporation] when the cells were plated *in vitro* in the presence of the double-stranded RNA (data to be published). The lack of antitumor activity of mouse interferon in the human tumor-nude mouse system may be interpreted to mean that in our test conditions the host-mediated resistance to the heterotransplant is not greatly affected by the exogenous administration of mouse interferon. To explain the lack of antitumor activity of human interferon, a variety of reasons could be considered, including the relatively rapid appearance and development of the tumors upon inoculation of the tumor cells (see Figs. 1–3). *In vitro*, both HT-1080 fibrosarcoma and A-375 melanoma cells were sensitive to the anti-proliferative effects of human (fibroblast and/or leukocyte) interferon (data to be published), so that their lack of antitumor activity *in vivo* could not be rationalized in terms of an abnormal resistance of the tumor cells to the "anticellular" effects of interferon.

The fact that neither human interferon nor mouse interferon exerted an inhibitory effect on tumor growth in our human tumor-nude mouse systems could obviously be attributed to the relatively low doses of interferon employed (5×10^5–5×10^6 U/kg). It should be pointed out, however, that these doses approach or even exceed those which would seem technologically feasible in human beings and that similar interferon doses have been found effective in inhibiting the growth of methylcholanthrene-induced tumors in mice [46].

It should be recognized that malignant human tumors when transplanted into nude mice generally grow as sharply circumscribed nodules, do not break through their compression capsules and do not metastasize. In this sense, they do not express their malignant potential in the environment of the nude mouse. This rather "benign" behaviour of human tumors transplanted into nude mice may eventually explain why interferon, unlike poly(I)·poly(C), failed to suppress tumor growth. One may indeed speculate that interferon predominantly acts by inhibiting spread of the tumor cells and formation of metastases. Therefore, any potential antitumor effects of interferon would go unnoticed if the host itself prevents metastasis.

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Letter to the Editor

Cyclophosphamide Pretreatment Reduces the Toxic Effect of High Dose Melphalan on Intestinal Epithelium in Sheep

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WE HAVE investigated binary combinations of cytotoxic drugs in mice which have less than the expected toxicity on normal tissues such as bone marrow [1–3], urothelium [2] and intestinal epithelium [3]. A priming dose of cytotoxic drug is given, followed by an interval, and then a large dose of either the same or a different cytotoxic drug. Very often the amount of drug that can be given in the large dose exceeds that which would normally be fatal to the mouse. The time interval between treatments is critical and varies from 2 to 4 days depending on the drugs used and the tissue(s) at risk.

Evidence has been presented [2, 3] that tumour tissue is not spared by drug combinations that spare normal tissues, and drugs used in this way have increased the therapeutic index. It was therefore important to see if a drug combination of this sort spared tissues in a large mammal such as the sheep, and to establish the optimum timing between priming dose and challenge dose. Sheep were chosen as they were readily available from local farms and the two drugs investigated were cyclophosphamide and melphalan as these have been extensively investigated [2, 3].

Barren ewes, kept for the duration of the experiment in a purpose built animal house, ranged in weight between 49 and 58 kg. Cyclophosphamide (cyclophosphamide monohydrate, Koch-Light Ltd.) was dissolved in saline; melphalan (Alkeran, Burroughs Wellcome Co.) was first dissolved in 1 ml of 2% acid alcohol and then in buffered diluent provided by the manufacturers. Both drugs were given i.v. as single injections. Cyclophosphamide, 10 mg/kg was always given first and followed at a variable time interval by melphalan, 3 mg/kg. The animals were killed 4 days after the melphalan with pentobarbitone (Expiral, Abbott Lab. Ltd.) and an immediate autopsy was performed. Blocks of tissue were taken at standard points in the duodenum, jejunum, ileum and ascending colon, fixed in 10% formol saline and processed for routine microscopy. Morphological changes in the intestinal mucosa were scored on a semi-quantitive 0 to 3 scale.

The effect of different time intervals between cyclophosphamide pretreatment and the dose of melphalan on the intestinal mucosa are summarised in Table 1. Histological examination of the gut epithelium in animals treated with melphalan alone (last column) indicated that few normal elements remained 4 days after this large dose of melphalan. With cyclophosphamide given 2 days before melphalan, the mucosal damage appeared to be even more severe. If, however, an interval of between 5 and 9 days was left between cyclophosphamide pretreatment and high dose melphalan, mucosal damage was much less pronounced, with the optimum when cyclophosphamide treatment preceded melphalan by 7 days.

Peripheral blood counts, measured at the time of killing (Table 2), indicated that the total leukocyte count fell less in animals pretreated with cyclophosphamide 7 days before melphalan.

Although no convincing explanation of the underlying mechanism has been established, the ability of a priming dose to reduce the

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Table 1. *Changes in intestinal mucosa in sheep treated with a sequence of* 10 mg/kg *cyclophosphamide* (*CY*) *and* 3 mg/kg *melphalan* (*M*) *given at different time intervals. Symbols above the diagonal lines refer to villi, below the lines to crypts*

Tissue	CY given 2 days before M	CY given 5 days before M	CY given 7 Days before M	CY given 9 Days before M	M Alone
Duodenum	+ + / + +	+ + + / + +	+ + / +	+ + + / + +	+ / +
Distal jejunum	0 / +	+ / + +	+ + + / + +	+ / +	0 / +
Distal ileum	0 / 0	+ / +	+ + + / +	+ + + / +	0 / +
Ascneding colon	/ + +	/ + + +	/ +	/ + +	/ 0

Changes in villi, small intestine:
0 = Completely absent.
+ = Flat with epithelial covering.
+ + = Raised with some epithelial covering.
+ + + = Condition approaching that seen in untreated control animals.

Changes in crypts, small intestine and large intestine:
0 = Completely absent.
+ = Dilated with incomplete epithelial lining.
+ + = Intact but abnormal.
+ + + = Condition approaching that seen in untreated control animals.

Table 2. *Effect of varying the timing between* 10 mg/kg *cyclophosphamide* (*CY*) *and* 3 mg/kg *melphalan* (*M*) *on the peripheral leukocyte counts in sheep*

	Treatment					
	Normal	CY given 2 days before M	CY given 5 days before M	CY given 7 days before M	CY given 9 days before M	M alone
24 hr after melphalan	8800 ± 809	N.D.	N.D.	4500 ± 312	N.D.	6357 ± 121
96 hr after melphalan		387 ± 137	1284 ± 149	2505 ± 100	865 ± 74	557 ± 63

Counts ± S.E./mm^3.
N.D. = Not done.

toxicity of a large dose of melphalan has been shown to operate in bone marrow in mice [3] and rats [4] and in the intestinal epithelium in mice [3] and sheep. This phenomenon is clearly not species or tissue specific, though the mechanisms may be different depending on the tissue.

If the phenomenon occurs in man, the timing and dosage will almost certainly be closer to that for sheep than for rats or mice, and it is unlikely that the timing for gut sparing in man will involve an interval of less than 5 days. Gut sparing would be the major objective in man since protection of the bone marrow is less of an advantage in centres where adequate support can be given during the period of drug-induced pancytopenia.

Experimental tumour systems studied so far [2, 3] have not been spared by binary drug combinations which spare normal tissues. Tumour cells presumably lack some of the

control mechanisms of normal renewal tissues and they may not have the capacity to alter their biochemical or proliferative behaviour after a priming dose of drug. By administering drugs in ways which reduce the toxicity of these drugs to normal critical tissues, larger doses may be given and an improvement in anti-tumor effect obtained.

Acknowledgements—We are most grateful to Dr. J. G. Hall and Dr. J. Reynolds for help and advice and to Mr. B. N. Hudspith and Mr. D. Glover for technical assistance.

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Letter to the Editor

Impairment of Inflammatory Reactions in Tumour-Bearing Mice as Measured by Evans Blue Extravasation*

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MANY authors have reported diminutions of skin reactions of delayed type hypersensitivity in cancer patients, sometimes in relation with the prognosis of the disease. Some investigations suggest that this effect could result from an impairment of the non-specific step of the response rather than from a failure in the function of antigen-reacting cells. Johnson *et al.* [1] observed that cancer patients with diminished responses to the hapten DNCB had a parallel diminution of inflammatory reactions against Croton oil, a non-antigenic irritant substance. Likewise, Bernstein *et al.* [2], in cancer bearing guinea pigs, found a diminution of skin tests after local administration of non-specific irritant or of specific recall antigens. However, immune cells responded normally *in vitro*: antigen-stimulated blood lymphocytes underwent blast transformation and synthetized skin reactive factor.

In the present work, inflammatory reaction was measured in mice, at different times after implantation of a solid tumour. The vascular component of the inflammatory reaction provoked by the intradermic injection of Freund adjuvant oil was measured, using a quantitative Evans blue test. The alterations recorded seemed related with the size of the tumour. However, as a strong hyperplasia of spleen and liver took place during tumour growth, we wondered whether this generalized inflammation could play a role in the diminution of the local inflammatory response to Freund adjuvant.

Female B6 D2 mice, 6 per group, were tested 1–4 weeks after i.m. graft of 10^6 Lewis tumour cells. For the test, 10 μl oil of Freund incomplete adjuvant (DIFCO) were injected in both ear pinnas. Twenty-four hours later, the mice received i.p. 0.2 ml of a 2.5 p. 100 Evans blue solution. Diazo dyes which bind plasma protein have been widely used to quantify capillary permeability [3] especially in inflammatory reactions [4]. Four hours after Evans blue injection, under ether anaesthesia, 30 μl blood were collected from the retroorbitary sinus, added to 2 ml saline and centrifuged. Then the animals were killed, tumour diameter and spleen weight measured. From each ear, the inflammatory site was removed with a punch biopsy. The skin disks thus obtained were dissolved in 2 ml Soluene 100 (Packard) within 90 min at 60°C. Blood supernatants and Soluene solutions, both containing Evans blue, were analyzed by spectrophotometry respectively at 605 and 615 nm.

As shown on Table 1, the decrease of Evans blue extravasation at the inflammatory site was significant from the 14th day after tumour graft. On the 28th day, the amount of dye per ear was 3 times lower than in the control. This diminution could not be ascribed to a parallel decrease of dye haemoconcentration. On the contrary Evans blue was more abundant in the blood of tumour bearing mice. The decrease in inflammatory response was roughly parallel with the increase in size of the tumour and of the lymphoid organs, here expressed through the spleen weight. The histological examination of spleen and liver did not show any malignant metastasis but only non specific proliferation of mononuclear cells.

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Table 1. Inflammatory reaction measured by Evans blue extravasation during the growth of Lewis tumour

Days after tumour graft	μg Evans blue ± S.E.		Tumour diameter ± S.E. (mm)	Spleen weight ± S.E. (mg)
	At inflammation site	Per 30 μl blood		
7	8.8 ± 2.7	12.9 ± 3.6*	2.7 ± 0.5	121 ± 22*
14	6.8 ± 2.7*	14.4 ± 2.0*	5.8 ± 1.3	187 ± 47*
21	5.0 ± 2.8*	13.8 ± 4.6*	13.5 ± 2.1	419 ± 34*
28	3.2 ± 1.3*	12.6 ± 2.4*	18.9 ± 1.5	706 ± 126*
Control (no tumour)	9.4 ± 2.5	10.0 ± 1.2	–	83 ± 15

*Significantly different from control groups at Student's *t*-test.

The mechanisms by which the presence of a tumour can provoke a diminution of inflammatory response may be discussed. Products synthetized from tumour cells in culture have been shown to inhibit the chemotaxis of inflammatory cells towards inflammation sites [5]. On the other hand tumour growth was shown to be often accompanied by inflammatory reactions developing either locally in contact with tumour cells [6] or at distance, giving rise to a stimulation of the reticuloendothelial system [7]. Several authors have described interactions between inflammatory foci located in different parts of the body: the previous induction of an inflammation was shown to diminish the response to the ensuing inoculation of irritant material [8, 9]. The intravenous injection of bacterial organisms inducing granulomatous reactions was shown to impair the present Evans blue test (in preparation). Thus, the inflammatory reactions associated to tumour growth could be sufficient to diminish the skin response against an inflammatory agent. Interactions between two distant inflammatory processes could result from a competition for the use of common cells or soluble mediators. Moreover, antinflammatory substances have been extracted from inflammatory exudates (reviewed in [10]). Such factors would represent a regulatory process of inflammation which, in the present case, would have overflowed from the local reaction and extended to the whole body.

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Letter to the Editor

Assessment of Response to Therapy in Advanced Breast Cancer

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

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In 1977, a project of the programme on clinical oncology of the UICC published a system of guide-lines recommended for use in clinical trials designed to assess the objective response of locally advanced or metastatic mammary cancer to treatment [1–6]. Since then it has become apparent that the rules for defining duration of remission are not entirely clear. In particular, patients achieving a complete objective regression may have, additionally, a period when only in partial regression. The following amendments to the original publications have been agreed.

Categories of response

Objective regression. (i) Complete response. The disappearance of all lesions determined by two observations not less than 4 weeks apart. (ii) Partial response. Fifty per cent or more decrease in measurable lesions and unequivocal improvement in evaluable but non-measurable lesions with no new lesions developing, determined by two observations not less than 4 weeks apart.

No change and progressive disease. These categories remain as defined previously.

Duration of response

It is recommended that: (1) The period of "overall response" be from the date of start of treatment to the date of first observation of progressive disease. (2) The period of "complete response" be from the date complete response was first recorded to the date of first observation of progressive disease. (3) In those patients who just achieve a "partial response", only the period of overall response should be recorded.

Thus, in patients achieving a complete objective remission the duration of this complete response should be distinguished from the longer overall response recorded in these cases.

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Announcements

THE SECOND INTERNATIONAL CONFERENCE ON THE ADJUVANT THERAPY OF CANCER

The Second International Conference on the Adjuvant Therapy of Cancer will be held in Tucson, Arizona 28–31 March, 1979, under the sponsorship of the Cancer Center Division of the University of Arizona. Drs. Sydney E. Salmon and Stephen E. Jones will serve as Conference Co-Chairmen. Abstract forms are now available; the deadline for submission of abstracts (prepared in the format of AACR/ASCO) is 1 December, 1978. For abstracts forms or further information, contact:

Dorothy Baker,
Conference Coordinator,
Cancer Center Division,
University of Arizona,
Tucson, Arizona 85724,
602-626-6044.

WORKSHOP ON CLONING HUMAN TUMOR STEM CELLS

A practical workshop on soft agar cloning methods for human tumor stem cells will be held at the University of Arizona, College of Medicine, in Tucson, 3–5 January, 1979, under the sponsorship of the Cancer Center Division. The workshop will be presented by Drs. Sydney E. Salmon, Anne Hamburger, David S. Alberts, Ronald N. Buick and colleagues.

Further information may be obtained by writing to the Cancer Center Division, University of Arizona, Health Sciences Center, Tucson, Arizona 85724, or by phoning Dr. Ronald Buick at (602) 626-6408.

EUROPEAN ORGANISATION FOR RESEARCH ON TREATMENT OF CANCER (E.O.R.T.C.)

Second Breast Cancer Working Conference, Experimental and Clinical Aspects, in Copenhagen, Denmark, 31 May–2 June, 1979.

Program Committee: H. T. Mouridsen, T. Palshof, J. Daehnfeldt and E. Engelsman.

The scientific program of the Conference will include recent experimental findings and clinical studies in primary and advanced breast cancer.

The Conference will include lectures by leading authorities, panels of invited guestspeakers and free communications. The number of participants is limited to 250 and dead line for registration is 15 March, 1979.

Please address inquiries to:
H. T. Mouridsen and T. Palshof,
Finseninstitute,
49 Strandboulevarden,
DK-2100 Copenhagen Ø,
Denmark.

Telephone: (01) 26 08 50, ext. 342.

RADIATION BIOLOGY IN CANCER RESEARCH

"Radiation Biology in Cancer Research" will be the topic of the 32nd annual Symposium on Fundamental Cancer Research to be held in Houston at the Shamrock Hilton Hotel 27 February–2 March, 1979. Subjects to be covered include responses at low doses; targets and effects; delayed effects in tissues; tissue effects not definitely attributable to cell killing; tumor biology; and potential applications of radiobiology to radiotherapy. Co-chairmen: Dr. H. Rodney Withers and Dr. Raymond E. Meyn.

For additional information: Stephen C. Stuyck, M. D. Anderson Hospital and Tumor Institure, Houston, Texas 77030, (713) 792-3030.

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Recent Journal Contents (1978)

International Journal of Cancer *October* 1978

Human Cancer

K.-O. Fresen, M.-S. Cho and H. zur Hausen: Recovery of transforming EBV from nonproducer cells after superinfection with nontransforming P3HR-1 EBV.

E. Ward, B. Paigen, K. Steenland, R. Vincent, J. Minowada, H. L. Gurtoo, P. Sartori and M. B. Havens: Aryl hydrocarbon hydroxylase in persons with lung or laryngeal cancer.

E. L. Wilson and E. Dowdle: Secretion of plasminogen activator by normal, reactive and neoplastic human tissues cultured *in vitro*.

J. H. Dean, T. R. Jerrells, G. B. Cannon, A. Kibrite, B. Baumgardner, J. L. Weese, J. Silva and R. B. Herberman: Demonstration of specific cell-mediated anti-tumor immunity in lung cancer to autologous tissue extracts.

S. Einhorn, H. Blomgren and H. Strander: Interferon and spontaneous cytotoxicity in man. I. Enhancement of the spontaneous cytotoxicity of peripheral lymphocytes by human leukocyte interferon.

E.-M. Rutanen: The circulating alpha subunit of human chorionic gonadotrophin in gynaecologic tumours.

T. Shimbo, J. Yata and H. Okada: Nonspecific activation of complement by leukemic cells.

Experimental Cancer

L. O. Arthur, C. W. Long, G. H. Smith and D. L. Fine: Immunological characterization of the low molecular weight DNA binding protein of mouse mammary tumor virus.

J. S. Rhim, D. K. Park, P. Arnstein, R. J. Huebner and W. A. Nelson-Rees: Neoplastic transformation of canine embryo cells *in vitro* by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

N. Okada and H. Okada: Activation of complement by spontaneous leukemic cells of AKR mice.

M. V. Pimm, A. J. Cook, D. G. Hopper, A. M. Dickinson and R. W. Baldwin: BCG treatment of transplanted rat tumours of spontaneous origin.

E. Wiener, J. Spira, S. Ohno, N. Haran-Ghera and G. Klein: Chromosome changes (trisomy 15) in murine T cell leukemia induced by 7, 12-dimethylbenz(a)anthracene (DMBA).

J. M. Friedman, P. J. Fialkow, J. East, J. I. Bryant and A. C. Salo: Longitudinal studies of chromosomal abnormalities and reticulum cell proliferation in New Zealand black mice.

J. M. Friedman, P. J. Fialkow, J. Bryant, A. L. Reddy and A. C. Salo: Neoplastic behavior of chromosomally-abnormal clones in New Zealand black mice.

N. Isakow, S. Segal, N. Hollander and M. Feldman: An immunoregulatory factor associated with spleen cells from tumor-bearing animals. I. Effect on tumor growth and antibody production.

N. Hollander, N. Isakov, S. Segal and M. Feldman: An immunoregulatory factor associated with spleen cells from tumor-bearing animals. II. Characterization of the cell populations involved in its production and release.

J. East, R. J. Tilly, M. Tuffrey and J. J. Harvey: The early appearance and subsequent distribution of murine leukaemia virus in NZB embryos.

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F. Wendling and P. E. Tambourin: Oncogenicity of Friend virus-infected cells: determination of spleen colonies origin by the H-2 antigens as genetic markers.

D. E. Onions, L. N. Owen and D. E. Bostock: Leukocyte migration inhibition responses in canine lymphosarcoma.

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G. Y. Gillespie, C. B. Hansen and S. W. Russell: Resurgence of killing and *in vivo* protection mediated by lymphocytes cultured from lymph nodes draining Moloney sarcomas.

B. M. Vose *et al.* Restricted autologous lymphocytotoxicity in lung neoplasia.

M. H. Jun *et al.* Enhancement and metastasis after immunotherapy of ovine squamous cell carcinoma.

P. Gaffney: Evaluation of the normal-lymphocyte-transfer test.

D. J. Flavell, A. Singer and C. W. Potter: Cell-mediated immunity to encephalitogenic factor (MMI test) in women with cervical dysplasia and carcinoma *in situ*: the effects of serum.

J. E. D. Dyson and P. J. Corbett: Effect of lymphocyte supernatants on the electrophoretic mobility of erythrocytes: significance in cancer diagnosis.

R. C. Miller and E. J. Hall: Oncogenic transformation *in vitro* by the hypoxic cell sensitizer misonidazole.

J. Ashby, J. A. Styles and D. Paton: Potentially carcinogenic analogues of the carcinogen hexamethylphosphoramide: evaluation *in vitro*.

J. A. DiPaolo, R. L. Nelson and B. C. Casto: *In vitro* neoplastic transformation of Syrian hamster cells by lead acetate and its relevance to environmental carcinogenesis.

R. J. B. King *et al.* Measurement of oestradiol receptors by five institutions on common tissue samples.

M. M. Roberts *et al.* Oestrogen receptors and the response to endocrine therapy in advanced breast cancer.

H. B. Marsden and W. Lawler: Bone-metastasizing renal tumour of childhood.

F. Gjerris *et al.* Incidence and long-term survival of children with intracranial tumours treated in Denmark 1935–1959.

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M. J. Embleton and P. T. Iype: Surface antigens of rat liver epithelial cells grown in medium containing foetal bovine serum.

A. R. Turnbull *et al.* Autoantibodies in early breast cancer: a stage-related phenomenon?

L. Dazord *et al.* Resistance to transplanted cancer in mice increased by live Brucella vaccine.

N. B. Atkin and M. C. Baker: Duplication of the long arm of chromosome 1 in a malignant vaginal tumour.

V. Covelli *et al.* Life-span and spontaneous tumours in mice with high and low antibody responses (Biozzo mice).

P. J. Dawson, A. H. Fieldsteel and J. McCusker: Incidence of spontaneous tumours in neonatally thymectomized rats.

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Papers to be Published

F. MERINO

Studies on the specificity of cytolytic antibodies to methylcholanthrene-induced sarcomas elicited by immunization of syngeneic mice.

J. J. STRAGAND, P. G. BRAUNSCHWEIGER, A. A. POLLICE and L. M. SCHIFFER

Cell kinetic alterations in murine mammary tumors following fasting and refeeding.

M. D'INCALCI, G. BOLIS, T. FACCHINETTI, C. MANGIONA, L. MORASCA, P. MORAZZONI and M. SALMONA

Decreased half life of cyclophosphamide in patients under continual treatment.

R. VERLOES, G. ATASSI, P. DUMONT and L. KANAREK

Tumour immunoprophylaxis exerted by the antimicrococcus immunity. II. Influence on the proliferation of murine plasmacytoma (MOPC 173) and Ehrlich carcinoma cells.

K. D. BAGSHAWE, R. M. LEQUIN, Ph. SIZARET and YU. S. TATARINOV

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Oestrogen receptors and peripheral serum levels of oestradiol-17β in patients with mammary carcinoma.

E. MOREL-CHANY, C. GUILLOUZO, G. TRINCAL and M.-F. SZAJNERI

"Spontaneous" neoplastic transformation *in vitro* of epithelial cell strains of adult rat liver: cytology, growth and enzymatic activities.

G. BADARACCO, M. MORELLI, C. NERVI and G. STARACE

Cytokinetic effects of continuous infusion of vincristine in head and neck carcinoma.

D. R. MORGAN, L. N. OWEN and D. E. ONIONS

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W. DE LOECKER and G. VAN DER SCHUEREN

In vitro effects of methotrexate, 5-fluorouracil, cyclophosphamide and vincristine sulphate on the metabolism of induced mammary cancers in rats.

R. D. BULBROOK, J. W. MOORE, G. M. G. CLARK, D. Y. WANG, D. TONG and J. L. HAYWARD

Plasma oestradiol and progesterone levels in women with varying degrees of risk of breast cancer.

M. LEYRITZ and J. H. JONCAS

The interaction of hydrocortisone, interferon and the Epstein–Barr virus in lymphoid cells.

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Erratum

The study presented in the article entitled "An assessment of the effects of steroid hormones and antiestrogens on short-term organ culture of human endometrial carcinoma", by S. Jacobelli, G. Sica, F. Ranelletti and G. Barile, in the *European Journal of Cancer* Vol. 14, No. 9, pp. 931–938, was supported in part by "Progetto Finalizzato C.N.R. Biologia della Riproduzione".

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Perspectives in Cancer Research

The Comparative Epidemiology of Selected Neoplasms Between Dogs, Cats and Humans. A Review

HOWARD M. HAYES, JR.

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THERE are a multitude of factors implicated in the etiology of cancer. Singly or in combination, determinants such as viruses, genetic susceptibility, alterations of endocrine metabolism, and exposures to solar radiation, X-radiation, certain chemicals, radioactive elements, inorganic substances, foods and drugs have been identified [1]. Yet, there is considerably more research to be done. Who is at risk for cancer, when and why? Some of the answers may lie in gaining new knowledge about disease processes in lower animals.

Proof of the kinship in the etiology of animal and human cancer is easily demonstrated by the work of Holsti and Ermala [2]. These investigators attempted to induce lung cancer in mice by painting their oral mucosa with tobacco tar. Rather than producing tumors of the lung, they induced carcinomas of the urinary bladder. The association of tobacco usage and subsequent bladder cancer in humans has now been established and quantified [3, 4].

The study of spontaneous occurring neoplasms in animals has recently gained further impetus. With the identification of excessive mortality rates of particular human cancers in specific U.S. counties [5], it now makes it possible to pursue the epidemiology of the lesion in similarly exposed pets. Dogs and cats which are kept as pets could be an ideal sentinel for monitoring the effects of low-level environmental contaminants. Pets share their owner's environment except for work-related exposures and tobacco usage. These two confounding variables presently bias many human surveys.

This review concerns the epidemiology of canine and feline neoplasms of the testis, ovary, mammary gland, urinary bladder, kidney, thyroid gland, chemoreceptor system, brain and blood–lymphatic system. Comparisons will be drawn between the human and veterinary epidemiology when possible.

TESTIS

The mortality rate from testicular neoplasms has doubled in men in the last 20 yr [6]. This lesion is now the leading cause of cancer death in the U.S. among men between the ages of 25 and 29 [5]. These neoplasms are second in hospital prevalence in the dog [7], but are very rare in the cat.

Men and dogs share many of the epidemiologic features of testicular tumors; the primary exception is the different frequency of occurrence of certain cell-types. Embryonal carcinomas and teratomas predominate in children and seminomas in adults, but Sertoli cell and interstitial cell tumors in humans are quite rare [6]. In the dog, seminomas, Sertoli cell tumors, and interstitial cell tumors occur with about equal frequency; teratomas have been unreported [7].

Several canine breeds have been identified with an excessive risk of one or more cell-types of testis including the Boxer, Weimaraner, Shetland Sheepdog and German Shepherd dog. What is intriguing is that the factor(s) responsible for the familial risk in Boxers expressed itself in three different tumor cell-types, whereas in the Weimaraner, just two cell-types were present excessively, and in the Shetland Sheepdog and German Shepherd dog only one cell-type [7]. In about half of the infrequent familial aggregates of testicular

neoplasia in man, more than one tumor cell-type was exhibited [8].

Other factors besides inheritance must play a large role in testicular tumorigenesis. There is some evidence to suggest that environmental exposures associated with rural or urban living contribute to the origin of seminomas in man [9–12]. Unfortunately, comparative data for dogs are not presently available.

Cryptorchism has long been recognized as a contributing factor to testicular neoplasia. Whether the etiology of the tumor(s) is founded only upon the dysgenetic gonad or is also the result of the inguinal and intra-abdominal environment is debatable [8]. Cryptorchid dogs have an estimated risk for testis tumors 13.6 times that of normal dogs [7]. In cryptorchid men, the relative risk has been estimated to be 14 by Mostofi [6] and 8.8 by Morrison [13]. However, tumor cell-types associated with the defect are different in each species. Among cryptorchid men, seminomas are the major cell-type [6, 13], while seminomas and Sertoli cell tumors share about equal risk in cryptorchid dogs [7]. Interstitial cell tumors have not been observed with cryptorchism in humans and only very rarely in dogs.

The Shetland Sheepdog has been proposed as an appropriate canine model for further research into testicular maldescent and tumorigenesis [7]. This breed has a predilection for the occurrence of each independent factor [7, 14], plus the concomitant occurrence of both [7].

OVARY

Ovarian malignancies represent 5% of the cancers diagnosed in U.S. women. Epithelial neoplasms account for about 75% of the U.S. incidence of ovarian cancer in white females [15]. Second most frequent are granulosa-theca cell tumors. In the largest canine study yet presented, epithelial neoplasms accounted for 60% and granulosa-theca cell tumors 27% of the classifiable ovarian tumors [16]. Overall, ovarian neoplasms are infrequently seen in the dog and cat.

Epithelial ovarian cancers, as a group, show consistently increasing incidence with age through 75 yr in women. In contrast, the incidence rates for malignant granulosa-theca cell tumors present two relatively static levels of occurrence with 45 yr being the age when risk increases [15]. Similarly in dogs, risk increases strikingly with age for bitches with epithelial ovarian tumors. Those with granulosa-theca cell tumors have a generally constant risk between 4 and 15 yr of age, followed by an increase [16].

There is a strong histologic similarity between ovarian neoplasms in dogs and those in women with the exception of granulosa-theca cell tumors. These tumors show considerably more morphological variation in bitch dogs than in women. This finding led Willis [17] to suggest that granulosa-theca cell tumors and epithelial ovarian tumors in the dog may share a common etiology. However, his hypothesis is not consistent with the reported epidemiology of the lesion [16].

There is little evidence of a familial association of ovarian neoplasms in women [18]. However, the incidence of ovarian cancer is much higher in white than black women. The difference here is attributed to the much higher incidence of epithelial ovarian cancer in whites over 45 yr of age than experienced by blacks [19]. Inheritance plays only a small role, at most, in the etiology of the canine lesion as the Pointer is the only breed identified with high risk for ovarian cancer [16].

The pathogenesis for spontaneous ovarian tumors in humans and animals is obscure. Suspected causes among women include hormonal imbalances and environmental carcinogens. Lingeman, in her treatise on human ovarian cancer [18], suggests the latter as the most prominent cause of the lesion, although few definitive clues exist. Unfortunately, the presently known epidemiologic features of canine ovarian cancer shed no new light on etiology [16]. Yet, the bitch dog does appear to be a suitable model for testing suspected ovarian carcinogens in women because of the similarities of the disease in both subjects.

MAMMARY GLAND

Tumors of the mammary gland are the most common neoplasm in the female dog but are infrequent in the cat [20]. The ratio of female to male dogs with the lesion is about 50:1, which is about the same ratio in people [21]. The distribution of mammary cancer by age in bitch dogs appears similar to that in women, using Lebeau's [22] dog-years to human-years formula [23].

Histologically, feline mammary carcinomas are more readily recognized as malignant than those from the dog. But occasionally in the cat, and often in the dog, mammary carcinomas are not uniform in histologic ap-

pearance. As in women, it appears that a correlation exists in cats between the histologic grading of malignancy and prognosis [24].

A relationship between tumor risk and parity in the bitch dog has been disputed [23, 25–27]. Schneider *et al.* [23] have reported a significant sparing effect for mammary cancer occurs in the bitch dog that had an oophorectomt prior to $2\frac{1}{2}$ yr of age. Studies of human breast cancer show that (1) risk increases with the age of the mother at giving first birth, (2) women under 30 yr of age who complete a full term pregnancy have less cancer risk than nulliparous women, although cancer protection is generally not enhanced by additional births [21], and (3) oophorectomy in women under 40 yr of age has a protective effect against the later development of breast cancer [28].

Familial factors do play a role in the etiology of the tumor. Female relatives of women with breast cancer have 2–3 times the risk for the lesion than the general population [21]. The increased risk is especially evident in the patient's children [29, 30].

In dogs, an excessive risk for mammary cancer has been identified in four canine breeds—Miniature Poodle, English Setter, German Shorthair Pointer and Pointer; it is important to note that the Pointer breed is also at high risk for ovarian cancer [18]. The occurrence of both cancers has been linked in some human families [31].

More investigation is needed to delineate factors possibly affecting canine and feline mammary cancer (i.e., obesity, the precursor effect of benign mammary neoplasms). Yet, there appears to be ample reason to use the dog and cat as models for research.

URINARY BLADDER

In the U.S. bladder cancer accounts for 4.3% of the incidence of all malignancies in humans [15]. In dogs, it represents 1% of the hospital prevalence of cancer [32], but is much rarer in cats [20]. Transitional cell carcinoma is the most common cell-type diagnosed in people [33] and dogs [32].

Four canine breeds (Scottish Terrier, Shetland Sheepdog, Collie, Beagle) show a marked propensity for the malignancy [32]. There is little evidence for an inheritance role in humans, although two families have been identified with an unusual aggregation of bladder tumors [34, 35]. One characteristic investigated in the living members of these families was their level of urinary tryphophan metabolites, a suspected cause of bladder cancer [33]. The similarities in the tryphophan metabolism between humans and dogs [36, 37] suggest that further studies using high-risk breeds of dogs could be helpful.

Female dogs have been detected with 1.5 times the risk of male dogs ($P<0.05$) for the tumor [32]. This sex differential probably occurs because of the infrequent urination, on a daily basis, by the female dog as compared to the male [38]. Urine-borne carcinogenic agents thus would have longer exposure to the bladder epithelium in the female than the male dog.

The sex risk is reversed in humans [15]. About one-tenth of the incidence of bladder cancer in men is reportedly associated with cigarette smoking [4, 39] and occupational exposures to chemicals, e.g., aromatic amines [33].

Recent canine trials indicate a dose–time relationship can also exist in the etiology. The total dose of carcinogen required to produce tumors is considerably less if given in small amounts over an extended period of time [40]. The latent period has been as long as 10 yr in some trials. Yet, once carcinogenesis has been initiated, removal of the exposure does not inhibit the growth of the tumor [41]. The overall low frequency of bladder cancer in dogs is consistent with a low dose response to general environmental exposures over an extended period of time [32].

It has been proposed that canine bladder cancer should be made a reportable disease in the U.S. [32]. Increasing case counts over time would possibly identify geographic areas where emerging environmental hazards to humans are now present.

KIDNEY

Very little is known about the etiology of renal carcinoma in animals and man. Wynder [42], in a review about the human lesion, proposed nutritional factors, e.g., animal fats and cholestoral, as a possible cause.

The epidemiology of feline renal carcinoma has been described. However, the recent description of canine renal carcinoma indicates an epidemiology very similar to that in humans [43].

Renal carcinoma accounts for 2% of human cancer in the U.S. [15]. In the dog, the tumor represents slightly less than 1% of

diagnosed malignancies. In neither species are genetic determinants conspicuous, although both men and male dogs are at increased risk for the lesion [15, 43]. And further, the male risk by age of canine renal carcinoma resembles the pattern of risk in men [43].

The reason for the male predominance of human renal carcinoma is obscure. Environmental factors are suggested by the rising incidence among American men, but not among women [44] and call for further studies into the role of tobacco, nutrition and occupational determinants. However, these factors cannot account for the male excess in dogs (and similar trends in sex ratio with age) and suggest the influence of host susceptibility, including the endogenous production or metabolism of sex hormones. This scenario is consistent with the evidence of remission in some human patients if treated with progesterone [45, 46].

THYROID GLAND

Endocrine malignancies compose 1.4% of all human cancers in the U.S. [15], 3.7 and 0.9% of the diagnosed cancers in dogs and cats [20]. Carcinoma of the thyroid is the predominant lesion among endocrine cancers. An environmental influence, linked with endemic goiter, has been disputed as an etiologic factor in human beings [47] and dogs [48]. X-radiation, a well-known etiologic agent for the human lesion [49], has not been implicated in the spontaneous neoplasia of the gland in domestic animals [48].

The morbidity and mortality from thyroid cancers is about $2\frac{1}{2}$ times greater in women than in men [5, 47]. Statistically, the diagnostic frequency in dogs shows no sex differential [48]. Familial aggregates of medullary thyroid carcinoma, singularly or in combination with other endocrine abnormalities, have been reported in man [50]. In dogs, breed predispositions for thyroid carcinoma have been detected in Boxers, Golden Retrievers and Beagles [48]. However, medullary thyroid carcinoma rarely occurs in dogs [51].

The excess risk among Boxer dogs is thought to be partly due to the anomalous follicular patterns commonly seen in their thyroid glands. More interesting may be the finding of excessively high risk among the Beagle breed. Investigators have reported an unexplained high prevalence of thyroiditis in several Beagle colonies used in laboratory research [52, 53]. This type of thyroiditis is indistinguishable from Hashimoto's thyroiditis in man [54], which may predispose man to thyroid carcinoma [55–58]. Follow-up studies of Beagles with thyroiditis could clarify this relationship.

Certain other primary neoplasms have been identified in humans and dogs as occurring in association with thyroid carcinoma. One is a syndrome called multiple endocrine adenomatosis, type 1, which is a dominantly inherited constellation of tumors composed of adenomas and carcinomas of several endocrine organs, plus occasionally multiple lipomas [48, 59–61]. Another syndrome is one that involves tumors of the thyroid and chemoreceptor system (chemodectomas) [62–63].

CHEMORECEPTOR SYSTEM

The chemoreceptor system consists of numerous morphologically similar glomera located throughout the body. In people, the larger clusters of cells have been named the glomus jugulare, glomus pulmonale, aortic body and carotid body.

Although the function and mode of action is not completely understood, the aortic and carotid bodies have been shown to be responsive to changes in the oxygen and carbon dioxide tensions, pH, and temperature of the circulating blood [64]. These bodies can initiate an increase in the depth, minute volume and rate of respiration via parasympathetic innervation. Also, via the sympathetic nervous system, they can increase the heart rate and elevate the arterial blood pressure. The human and canine chemoreceptor systems are quite similar.

Chemodectomas are uncommon in humans and dogs and very rarely occur in cats. In people, they occur twice as often in the carotid body than other sites [65]. Conversely, about 80% of the chemodectomas in dogs are located at the aortic body [63]. Chemodectomas, regardless of location however, are histologically indistinguishable. There is usually no correlation between histologic appearance of this tumor and its malignancy potential [66].

The number of human chemodectomas observed in each sex has been about the same with one exception; Peruvians who live in the Andes experience a 6-fold excess among women [65, 67]. In dogs, males have about 2 times the prevalence of females [68]. In neither humans nor dogs are the reasons for this sex differential known. However, an association of seminomas and interstitial cell tumors in con-

junction with canine chemodectomas has been reported [63, 69].

Some of the determinants in the etiology of chemodectomas are known. Genetic factors have been implicated, by familial aggregations, in about 8% of the lesions in people [67, 70]. Further, chronic hypoxia seems to be implicated, at least among the Peruvians living at high altitudes [65].

In dogs, only breeds of brachycephalic English Bulldog family (i.e., Boxer, Boston Terrier) have been detected with significantly high risk [68]. These breeds frequently have soft tissue anomalies of the oral and nasal cavities which restrict and impede respiration [71, 72]. Yet, other brachycephalic breeds, such as the Pekingese and Pug, show no affinity for chemodectomas. Thus, for Bulldog breeds, a genetic predisposition plus an aggravation of respiratory distress may be necessary factors responsible for the tumorigenesis of their chemoreceptor system [68].

BRAIN

Intracranial neoplasms have held a special interest for researchers over many centuries. Irrespective of the tumor's behavior (benign or malignant), these neoplasms have an insidious effect caused by the increase in intracranial pressure due to tissue displacement.

The annual age-adjusted incidence of brain tumors in humans is about 5 per 100,000 in the U.S. [15]. These tumors are rare in domestic animals with the exception of the dog [73]. Gliomas, astrocytomas, and glioblastomas are the predominant tumor types of the brain in adult humans and dogs. Medulloblastomas predominate in children. Little is known about the types of brain tumors that occur in puppies.

The age distribution in humans is bi-phasic. The childhood peak is under 10 yr of age and the adult peak is 64–69 yr [15]. The peak risk in dogs occurs between 10 and 14 yr of age [73], the median of which approximates the second peak in humans [22]. White men have more brain tumors than white women, however no sex differential is evident in U.S. blacks [15]. No sex risk has been noted in dogs [73].

The etiology of naturally occuring brain tumors is obscure. An association of reticulum cell sarcoma in the human brain following renal transplant and immunosuppression has been observed [74]. In dogs, gliomas have been induced with Rous sarcoma virus [75].

Familial excesses for astrocytomas have been reported in people [76, 77]. In dogs, only breeds of English Bulldog ancestry show high risk [73]. As mentioned before, this composite dog family commonly have soft tissue anomalies of the nose and mouth which may result in chronic hypoxia.

Chronic hypoxia in humans can be a stimulus for fibrillary gliosis in the neonatal and infantile brain [78], the severity of which appears to be correlated with the length of time of prolonged hypoxia [79]. Gliosis may be a precursor of gliomas and other related tumors. Or, the conditions may share a common etiology (i.e., chronic hypoxia), since the entire spectrum of nodular and diffuse gliosis, gliomatosis, astrocytomas, and glioblastoma has been concurrently observed in people [80–82].

BLOOD–LYMPHATIC SYSTEM

Tumors of the hematopoietic system are the most common neoplasms diagnosed in cats and rank fourth in occurrence in dogs. The leukemia–lymphoma complex ranks first in cats and dogs for the percentage of malignancies [20]. Leukemia accounts for about half of the incidence of cancer in children under 5 yr of age. Lymphomas account for about 3% of the cancers in the same age group. Each lesion represents 3.25% of the annual age-adjusted incidence of cancer in the U.S. [15]. The age distribution of feline lymphosarcoma is biphasic [83] and similar in age pattern to human leukemia [15].

Lymphoid tumors constitute most of the hematopoietic neoplasms in dogs and cats, and lymphosarcoma is the most common form of malignancy of the system. It should be noted that the term "leukemia" in the veterinary literature has often been used as a general term to represent, in fact, lymphosarcoma. Histologically, lymphosarcoma in the dog and cat are similar and resemble the disease in man.

The Boxer [84–86] and English Pointer [86] have been identified with significantly high risk for canine lymphosarcoma. No sex-specific features are evident in the dog [86]. Unlike feline lymphosarcoma, transmission of the canine disease has been unsuccessful with cell-free distillates of the tumor [87].

Lymphosarcoma is the most prevalent malignant tumor in cats. No breed predisposition has been established. Although, males appear

to have a 2-fold risk compared with females [83].

The etiology of the feline lesion is a C-type RNA virus [88], commonly referred to as feline leukemia virus (FeLV). FeLV has been transmitted by cat-to-cat contact in the laboratory [89]. This is also the likely mode of transmission in the general population [90]. The errant and often hostile instincts of the male (non-castrated) cat to other male cats may subject them to sufficient cat-to-cat contact that could explain their 2-fold risk for FeLV infection.

Feline lymphosarcoma has been associated with at least one other infectious disease, feline infectious anemia (FIA). FIA is caused by an infection with Hemobartonella felis, a protozoan, and can result in severe anemia.

Cats with acute clinical expression of FIA have 12 times the risk of development of feline lymphosarcoma as matched controls [91]. The exact association between FIA and feline lymphosarcoma or between severe anemia and subsequent lymphosarcoma is unclear [92]. As in experiments with rats and mice [93, 94], severe anemia may trigger clinical lymphosarcoma in the cat. However, the causal organisms of FIA and FeLV could, in some way, be linked. Similar to lymphosarcoma, FIA appears to be naturally transmitted by cat-to-cat contact; male cats have a 2.5 fold risk for acute clinical disease [95].

Evidence has been presented to show FeLV can cross species barriers. Newborn and young puppies inoculated with FeLV will develop lymphosarcoma [96, 97]. Of great importance is the fact that FeLV will replicate in human cells [98, 99]. Many studies have evaulated the possibility of cat-to-human transmission of FeLV; none have shown the FeLV is a hazard to man [100].

CONCLUSIONS

Recent data about the epidemiologic features of 9 different tumor sites in dogs and cats have been reviewed. Far more information is generally known about cancer that occurs in dogs than in cats. The epidemiologic features for canine tumors at most organ sites show marked similarity to those in humans. Although, the frequency of particular cell-types at some organs often differs. The similarities in risk by age and sex should encourage further research to determine which cancers in the dog are correlated with environmental factors that affect human beings. Identification and monitoring the frequency of these canine cancers may provide a prognostic resource about subsequent environmentally influenced cancer in human beings.

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The Effect of Irradiation on the Rate of Oxygen Removal in the Lewis Lung Carcinoma

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Abstract—*The spatial distribution of oxygen removal rate, Q_R, has been measured in the Lewis lung carcinoma at intervals up to* 10 days *after a single exposure to* 2000 rad ^{137}Cs *γ-rays and also in untreated tumours allowed to grow to different sizes. The measured patterns of Q_R were compared with the histological structure of the tissue. There was a good correlation between Q_R and the distribution of viable and necrotic tissue within the tumour and Q_R towards the centre of large necrotic zones was as low as* 10% *of that of viable regions. A large increase in the proportion of necrotic tissue was observed in irradiated tumours particularly in the central region and this occurred during a period when the total volume remained approximately constant. Because of the increased necrosis, the distribution of oxygen utilization and the pattern of future growth both differed in irradiated tumours from those of untreated tumours of the same size. Following irradiation the average Q_R for central regions decreased to about* 30% *of the control level at* 5 days *and then showed a slow increase. The decrease in average Q_R was mainly a result of the increased proportion of necrosis. The Q_R of viable tissue alone showed much less change following irradiation although it was reduced to* 80% *of the control value at* 5 days.

INTRODUCTION

THE RATE of oxygen removal by a tissue, defined as the ratio of the rate of oxygen uptake per unit volume to the oxygen solubility of the tissue [1], is an important parameter of tissue oxygenation which, with the oxygen diffusion coefficient controls the total distance of penetration of oxygen into the tissue from capillaries. Significant spatial variations in oxygen removal rate have been demonstrated within a number of different tissues and have shown some correlation with regions of distinct histological appearance, such as for example the cortex and medulla of rat kidney and the viable and necrotic zones of an experimental tumour [2]. In addition, variation in oxygen removal rate may also occur within regions of tumour tissue having a uniform histological appearance.

In tumours which have been irradiated a number of factors may combine to produce changes in the oxygen removal rate. A transient change in oxygen removal rate may occur during the reorganization of a tissue containing a large number of cells killed by radiation as dead cells or cellular fragments are cleared from the tumour via the vasculature and the tissue is repopulated by surviving cells. Major alterations in the structure of a tumour, such as the formation of necrosis, which may result from radiation damage may be associated with less transient changes in the oxygen removal rate. Hence, changes in the distribution of oxygen removal rate in irradiated tumours can only be fully interpreted if changes in tumour structure are also taken into account. A detailed study of this kind should lead to an improved understanding of the role of tissue oxygen uptake rate in the response of tumours to fractionated radiation schedules, and the hypothesis that changes in oxygen uptake rate are a significant factor in the reoxygenation of tumours containing radioresistant hypoxic cells [3, 4] can be investigated.

A study of the effect of irradiation on the

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oxygen removal rate of the rat fibrosarcoma, SSB1a, has been reported previously [5]. The method has now been used for the Lewis lung carcinoma in the mouse. The properties of the Lewis lung carcinoma have been found to differ from those of the SSB1a fibrosarcoma both in terms of changes in the oxygen removal rate and changes in the tumour structure produced by exposure to radiation. These changes are discussed in terms of cellular processes occurring within the irradiated tissue of the two tumours.

MATERIALS AND METHODS

1. *Experimental procedure*

The method for measuring the distribution of the rate of oxygen removal in tissue has been described in detail previously [1]. A sample of recently excised tissue is mounted in a Perspex cuvette with a plane cut surface pressed against a 12 μm polythene membrane. The sample is pre-equilibrated with pure carbon monoxide for 5 min to saturate any haemoglobin in the tissue. It is then placed in a measuring chamber where it is allowed to equilibrate with a gas of known oxygen tension. The cuvette is then brought into contact with the plane end of a multicathode polarographic electrode which cuts off the oxygen supply to the respiring tissue and the rate of oxygen removal in a small zone of tissue adjacent to each cathode is calculated from the time course for the change in surface oxygen tension. The complete measuring system is installed in a temperature controlled cabinet maintained at 25°C.

2. *The definition of oxygen removal rate*

The technique measures the oxygen removal rate, Q_R in units of mmHg/sec, defined as the ratio of the rate of oxygen uptake to the oxygen solubility per unit volume of tissue and calculated from the equation

$$Q_R = \frac{\pi P_o}{8} \cdot \left(\frac{k}{i_o}\right)^2 \qquad [1]$$

where P_o is the partial pressure in mmHg of the equilibrating gas, k is the slope of the cathode current time course plotted against the square root of time and i_o is the intercept of this curve on the current axis. Time is measured from the moment of contact between the electrode and cuvette. Q_R is integrated over a tissue zone approximating to that of a cylinder of radius and length about 300 μm situated perpendicularly to the tissue surface [6]. Ninety-one concurrent measurements of Q_R are derived from separate cathodes positioned in a hexagonal array with 500 μm spacing. A computer is used to process the data and, by interpolation between individual data points, to plot a map of the distribution of Q_R within the total area of approximate diameter 5 mm sampled by the electrode (for example, Figs. 7 and 8).

3. *Treatment of tissue samples*

A special method is used for processing the tissue samples so that the structure can be compared exactly with the measured distribution of Q_R [2]. The tissue surface is held flat throughout the stages of fixation and wax embedding and the sample is sectioned within about 100 μm of this surface. The position of the electrode during the measurement is recorded by indian ink markers placed in the tissue. A grid consisting of a hexagonal array of points is placed over the tissue section so that the positions corresponding to individual cathodes can be located (Figs. 7 and 8).

The Lewis lung carcinoma was maintained by serial transplantation in C57Bl/10 male mice. At about 10 days after transplantation the tumours reached a mean diameter of 8.5–9.5 mm (measured parallel to the skin: the depth perpendicular to the skin was usually less than this) which was the standard size for irradiation. The tumours were irradiated by two opposed caesium-137 sources and were excised at appropriate times subsequently. Non-irradiated tumours at the standard size were used for controls. The tissue cuvettes held samples up to 8.5 mm diameter. In tumours of larger diameter it was necessary to select an area of appropriate size from the cross section of the cut tumour. This could be either in the centre of the tumour or in an eccentric position tangential to one edge which gave a more even distribution between viable tumour tissue and the necrosis which was increased towards the centre.

4. *Scoring of histological structure*

The structure of the Lewis lung carcinoma is that of patches of viable tissue interspersed with necrosis. A scoring system was devised to characterise the tissue structure in the neighbourhood of each cathode measuring position. Each point on the grid placed over the tissue section was centred in turn in the 500 μm diameter field of view of the microscope. The diameter of the field of view was

chosen to somewhat exceed the size of the zone corresponding to the measurement of Q_R to allow for any slight inaccuracies in the lining up of the grid and the original electrode position. The tissue viability was scored as a proportion of the area within each field of view according to the scale:

V > 95% viable tissue.
IV > 75% viable tissue.
III—25–75% viable tissue.
II < 25% viable tissue.
I < 5% viable tissue.

Non-necrotic tissue had a different appearance at certain times after irradiation from that in non-irradiated tissue due to the presence of nuclear fragments and many large multinucleate cells (Figs. 4 and 5). It was still distinguishable from the necrotic areas which contained cellular fragments and frequently zones of haemorrhage.

5. *Accuracy of the measurement* Q_R

The use of a cuvette membrane can introduce an error into the measurement of Q_R the size of which is a function of the ratios of the oxygen diffusion constant and the solubility between the membrane and the tissue. The error has been estimated to be less than 15% for most tissues [6] but can be assessed for a given tissue by comparing measurements made with and without a cuvette membrane.

Measurements on 4 Lewis lung carcinomas with differing Q_R distributions following equilibration with 50% O_2 and made with and without a 12 μm polythene membrane are given in Fig. 1. There is a small shift to lower Q_R values particularly at the highest Q_R values but this is estimated to be less than 15%. The difference between the means of the distributions is certainly less than 15%.

It has also been calculated that for a polythene membrane the error in Q_R is relatively independent of the value of Q_R itself [6]. Comparisons of relative Q_R values will therefore be significant even for differences of less than 15% for distributions measured within individual tumours and for differences between tumours of the same line. Some caution may be appropriate in interpreting differences between control and irradiated tumours since it is not known if a change in either the tissue diffusion constant or solubility for oxygen is produced. Some of the variation in Q_R within individual tumours could be due to variation either in the tissue oxygen diffusion constant which would affect the error on the estimate of Q_R, or in the oxygen solubility which would affect both the error in Q_R and the absolute value of Q_R. However, there is little information as yet about small scale variation in the oxygen diffusion parameters of tissues.

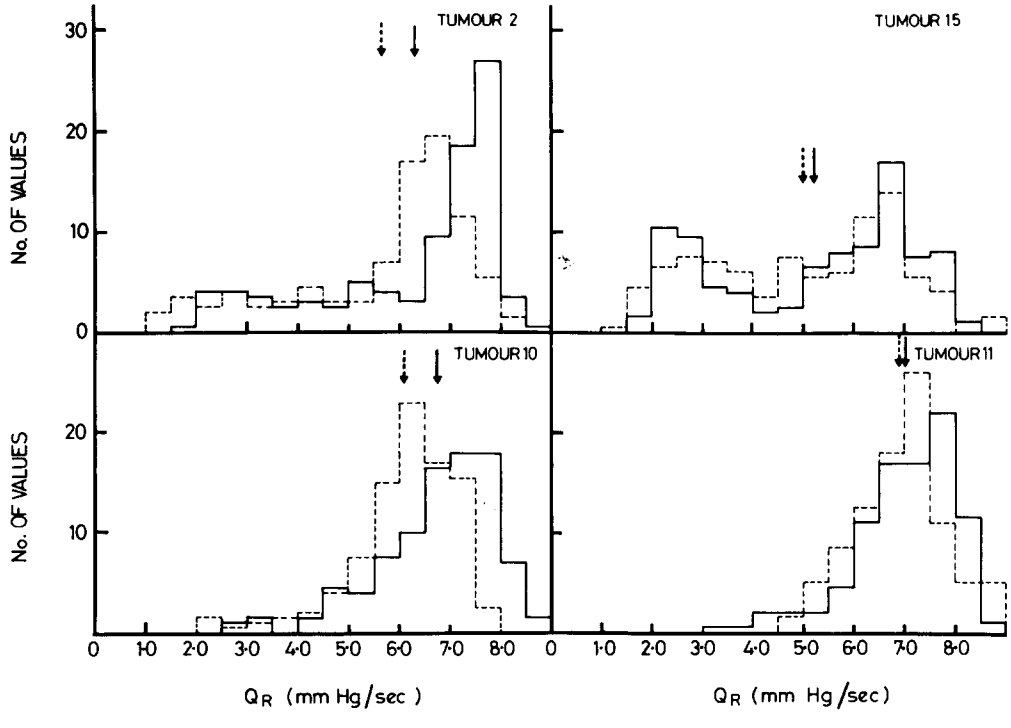


Fig. 1. Frequency distributions of Q_R *values for four samples of the Lewis lung carcinoma each measured with (solid line) and without (broken line) a* 12 μm *polythene membrane. Vertical arrows indicate the mean* Q_R *values.*

RESULTS

1. *Change in average* Q_R *in tumours exposed to* 2000 rad

The average change in Q_R for tumours exposed to 2000 rad Cs γ-rays is presented in Fig. 2. An average value for each tumour over the total area sampled was obtained by averaging 91 individual values from a single measurement. These values were again averaged within groups of similarly treated tumours to give the overall means and standard errors shown in the figure. The number of tumours in each group, shown in the figure, was at least 5 in most cases. The average Q_R values are for samples taken from both central and off-centre regions of the tumours and represent the accumulated results of more than one series of experiments. The measurements for central regions were made following equilibration with air whereas the off-centre samples were equilibrated with 50% O_2 to give a slight increase in accuracy for the measurement of Q_R in the areas containing a high proportion of viable tissue. The mean tumour volumes are also shown in Fig. 2. The tumour volumes remained rather constant during the first 6 days after exposure to 2000 rad except perhaps for a small increase in volume up to day 3. After the sixth day the tumours started to regrow but in an irregular fashion as indicated by the increase in the standard errors of the mean values. At later times the tumours tended to ulcerate so that measurements were not made after the tenth day.

There was a large difference between Q_R measurements for central and off-centre tumour areas. The Q_R for central areas decreased sharply to reach a minimum at day 5 of about 30% of the control value and then started to increase slowly. The Q_R for off-centre sampling areas decreased during the first 3 days to reach a plateau level at about 75% of the control level.

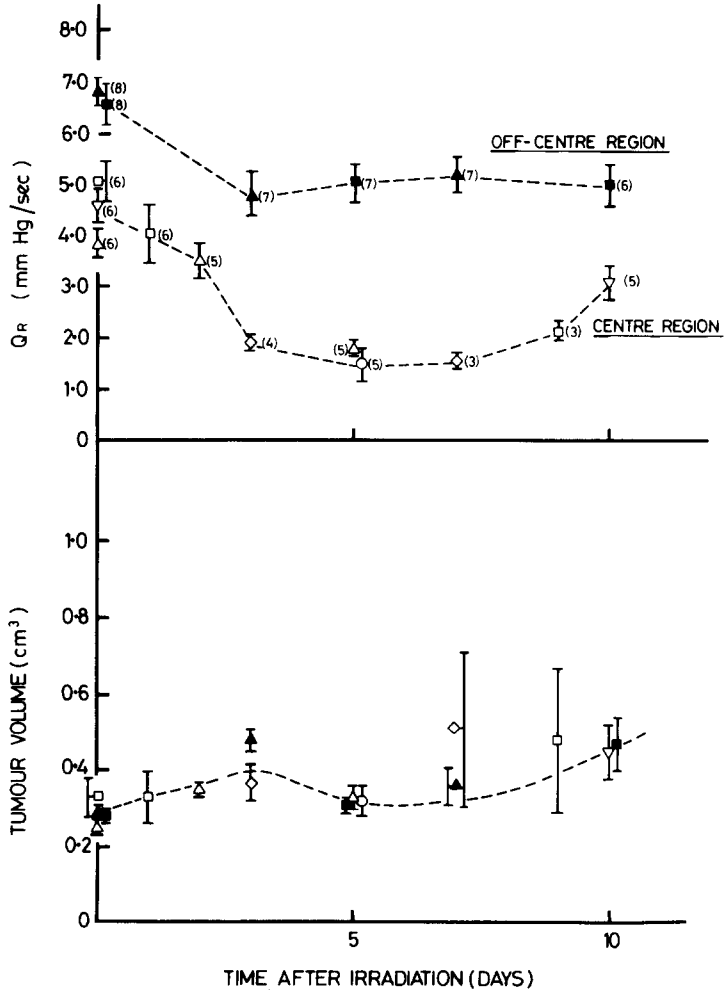


Fig. 2. The average Q_R *for central and off-centre regions and the tumour volume at various times after exposure to* 2000 rad. *The points represent the means and standard errors of the average* Q_R *values for the numbers of tumours indicated in brackets. Different symbols represent different batches of tumour implants.*

The large differences between the Q_R of central and off-centre samples are due to the greater proportion of necrotic tissue in the central samples (see No. 3 of Results). Similarly the smaller difference between the control levels for central and off-centre samples is due to the smaller amount of necrosis in the control tumours.

2. *Change in average* Q_R *in untreated tumours*

The mean Q_R for similar groups of untreated tumours was measured at selected times after reaching the standard irradiation size. The measurements were made on central sampling areas following equilibration with air and are shown together with changes in tumour volume in Fig. 3.

The tumour volume increased by about a factor 3 in 6 days during which time the mean Q_R decreased to about 50% of the control level. The change in Q_R was not as rapid or as large as that in irradiated tumours (Fig. 2) in which the volume did not change.

3. *Comparison of* Q_R *of irradiated tumours with histological structure*

Although the tumour volumes remained almost constant during the first 7 days after exposure to 2000 rad major changes in tumour structure took place during this time. These were assessed from a series of histological preparations of complete tumour cross sections. In control tumours, at the standard irradiation size, the proportion of necrosis was less than 5% of the total tumour area but this was increased to 30% in tumours which had been irradiated 7 days earlier. The proportion of necrosis was somewhat less than this in tumours sampled at later times after irradiation. At 7 days, the time of maximum necrosis, the viable tissue in the tumour was mainly confined to a shell round the periphery. At later times the tumours showed evidence of patchy regrowth with zones of viable tissue and necrosis rather randomly intermixed.

The Q_R was measured on batches of tumours sampled at 3, 5, 7 and 10 days after

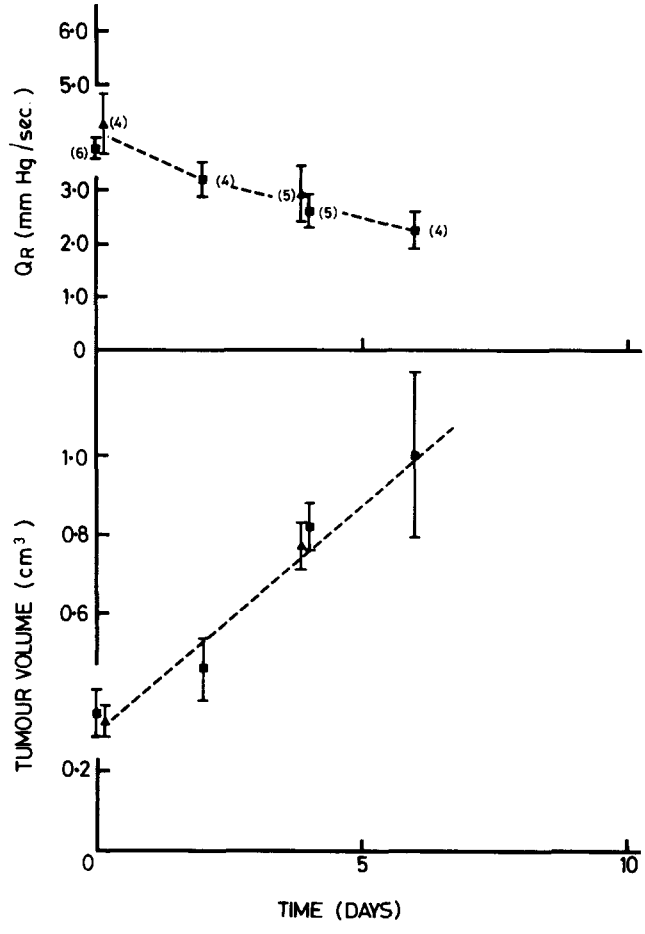


Fig. 3. The average Q_R *for central regions and the tumour volume for untreated tumours at various times after reaching the standard irradiation size. The points represent the means and standard errors of the average* Q_R *values for the numbers of tumours indicated in brackets. Different symbols represent different batches of tumour implants.*

2000 rad and histological sections were produced which showed the area sampled by the electrode. The areas of tissue beneath individual cathode positions were classified in terms of the proportional content of viable tissue according to the scale given in the methods (No. 4, Materials and Methods). The zones which were classified as viable contained many radiation-damaged cells at 3 and 5 days after irradiation (Fig. 5) but by 10 days the damaged cells had disappeared and the viable areas resembled those of control tumours (Fig. 4).

For each tumour the Q_R values corresponding to a given score for the viability index were averaged and the mean Q_R values for each viability index were averaged between all tumours in the group to give an overall mean and standard error. If a tumour had 5 or less values of Q_R for a given viability index this was not included in the mean for the group, but this only occurred for indices of I or II in control tumours where the proportion of necrosis was low. The results, shown in Fig. 6, were from the same batches of tumour that gave the mean Q_R values shown in the upper of the two curves in Fig. 2 for sampling from off-centre tumour regions. The Q_R of totally viable areas scored as V was found to be more than three times that of totally necrotic areas scored as I, and intermediate viability indices corresponded to intermediate values of Q_R.

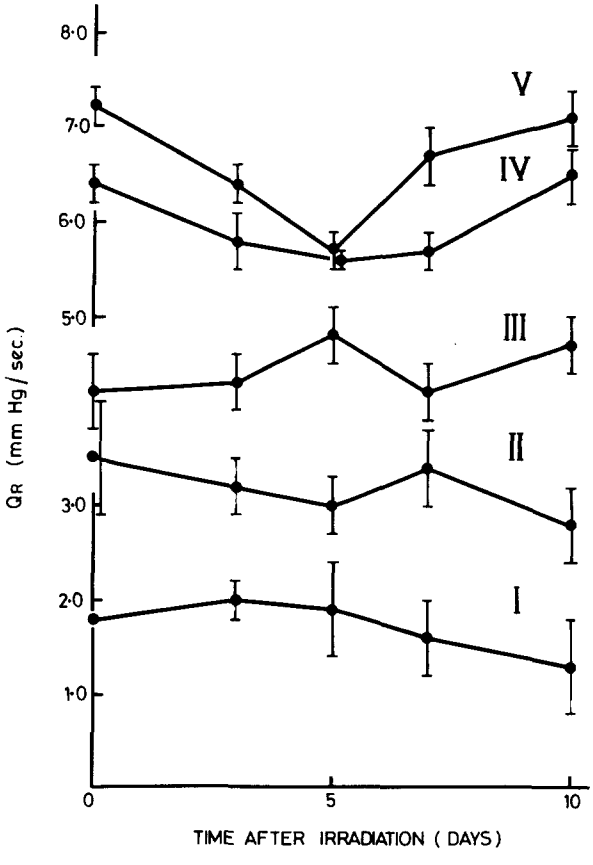


Fig. 6. Values of Q_R *for regions of viability indices* I–V *at various times after exposure to* 2000 rad. *The points represent the means and standard errors of the average* Q_R *values for each index for a group of tumours.*

Areas with a viability index of V were the only ones in which Q_R showed a significant change with time after irradiation. The Q_R decreased to 80% of the control level 5 days after irradiation and then increased almost completely back to the control level by day 7. Although the mean Q_R of areas with index I did not change significantly there was a clear tendency for Q_R to be less than 0.8 mmHg/sec towards the centre of the large necrotic zones which were found in tumours sampled at the later times after irradiation. In these cases the Q_R varied by more than a factor of 10 between the wholly viable areas and the centres of necrotic areas. An example of this is shown in Fig. 8 where the Q_R distribution for an area of tumour, sampled 10 days after exposure to 2000 rad and containing a large necrotic area, is compared with a photograph of the tissue section with a superimposed grid marking the area sampled by the electrode during the measurement. A similar diagram showing the Q_R distribution and corresponding histological structure for a control tumour is given in Fig. 7.

DISCUSSION

The Lewis lung carcinoma has been described as an anaplastic epidermoid tumour with a marked tendency to become haemorrhagic [7]. Small tumours usually have a rather uniform appearance but an increasing proportion of necrosis in larger tumours can lead to the restriction of intact tissue to thin shells surrounding zones of non-proliferating cells and to ulceration through the skin. The proportion of necrosis within the tumour is somewhat lower in tumours which become closely attached to the skin during growth. The increase in the proportion of necrotic tissue in larger tumours is associated with a reduced growth rate and with some changes in the kinetics of cellular proliferation [8, 9]. Areas of haemorrhage often develop in the neighbourhood of necrotic zones and may be caused by the deficient structure of many of the blood vessels. For example vascular spaces without an intact endothelial lining are found in the periphery of the tumour and these allow blood to come into direct contact

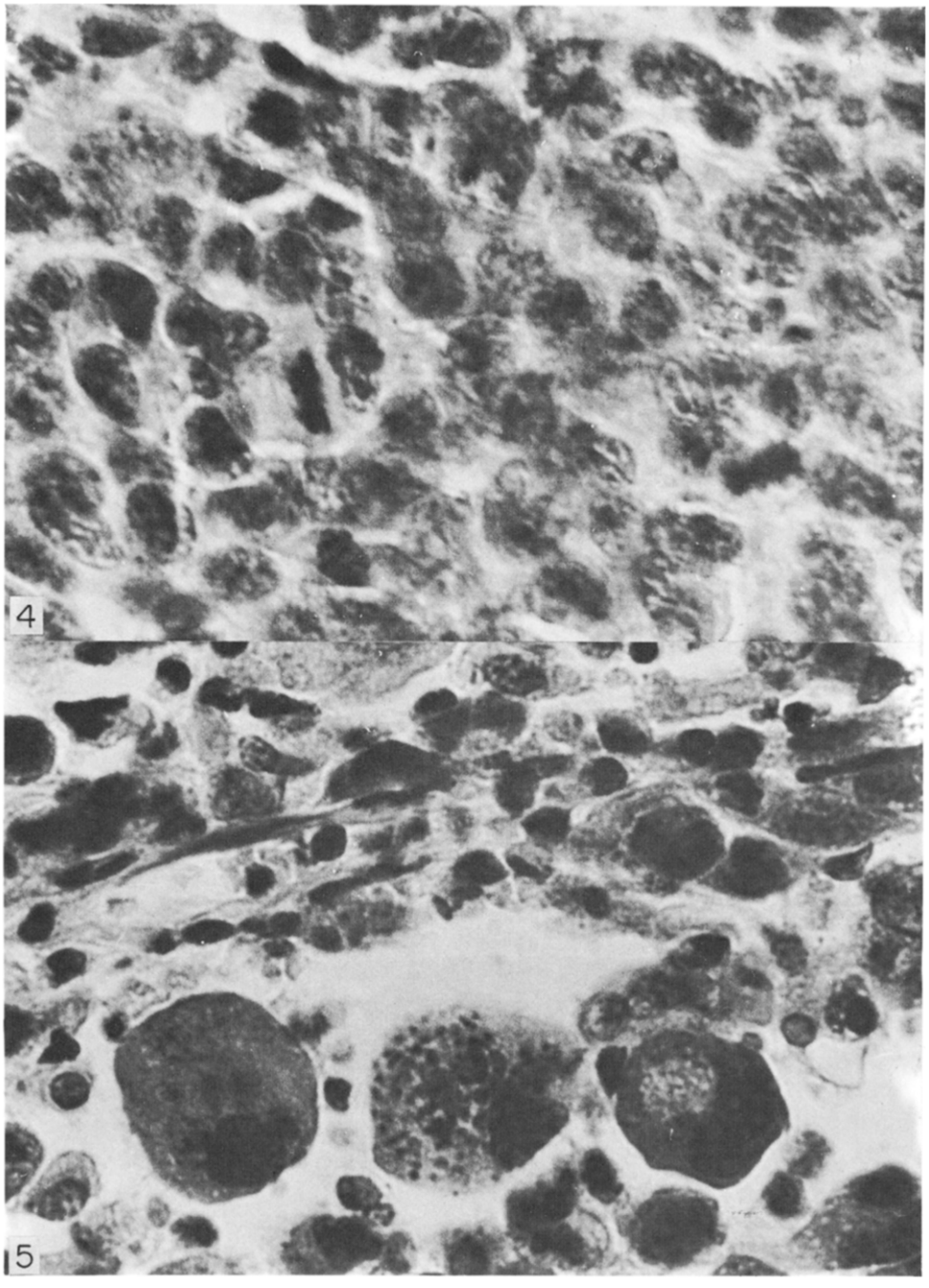


Fig. 4. *Region of a control tumour scored with viability index* V (× 1000).

Fig. 5. *Region of a tumour* 5 days *after exposure to* 2000 rad *scored with viability index* V. *Many radiation-damaged cells are present* (× 1000).

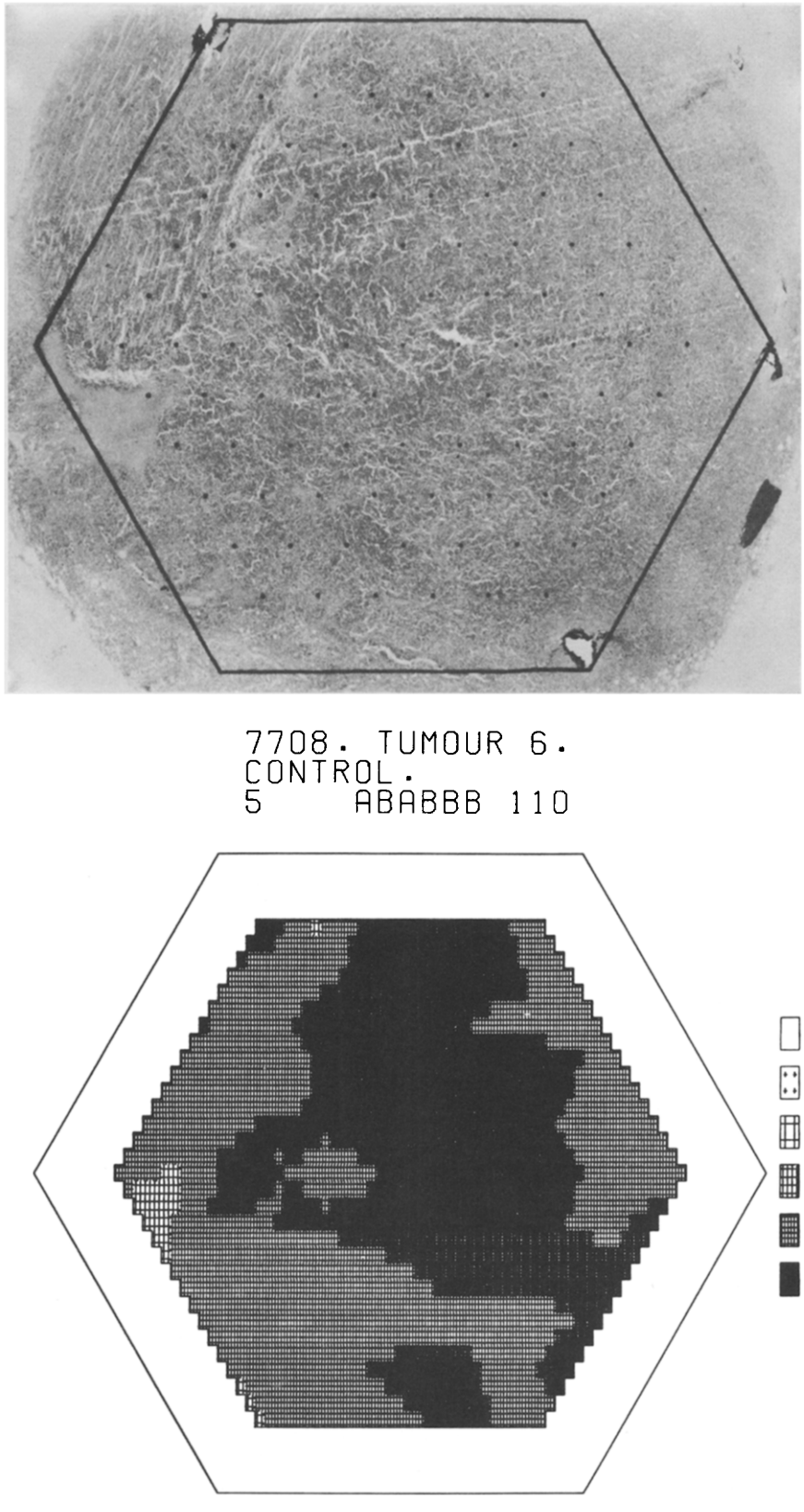
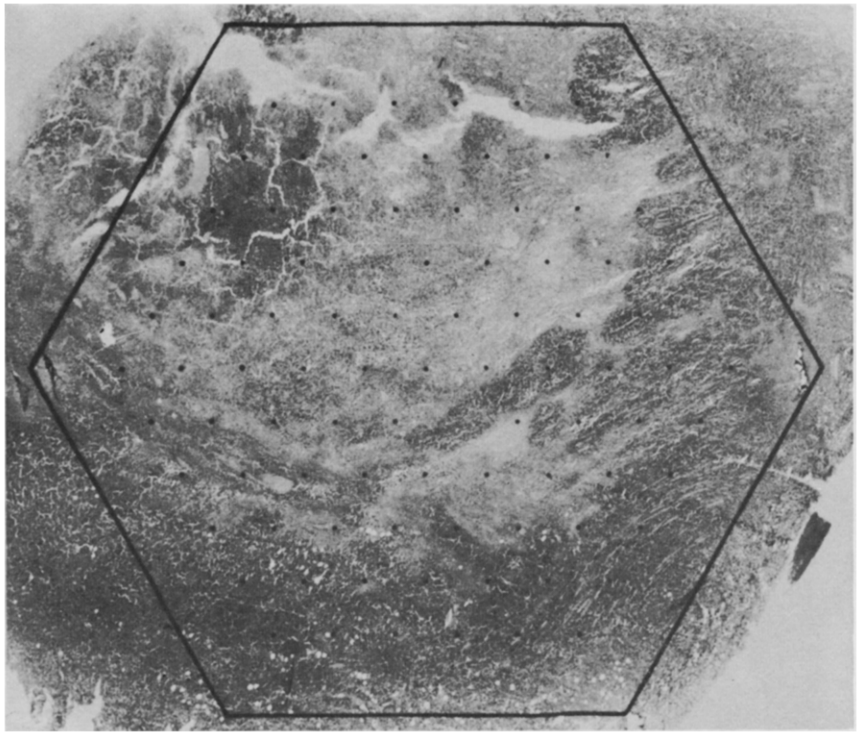


Fig. 7. Section of a control tumour with a hexagonal grid showing the positions of the electrode outline and cathodes during the measurement of Q_R *together with a computer-produced map showing the distribution of* Q_R. *The key shows the ranges of* Q_R *in units of* mmHg/sec. *The spacing between the cathode positions is* 500 μm.



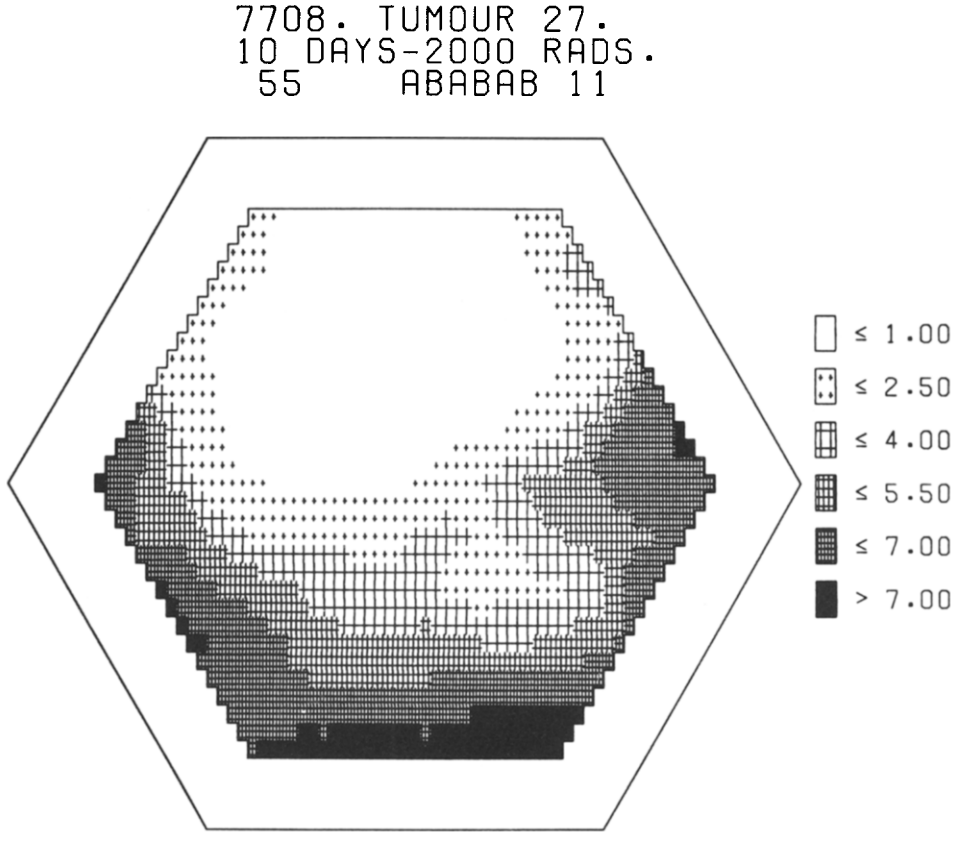


Fig. 8. Section of a tumour sampled 10 days *after exposure to* 2000 rad *and including a large necrotic area. The hexagonal grid shows the positions of the electrode outline and cathodes during the measurement of* Q_R *and the computer-produced map shows the distribution of* Q_R. *The key shows the ranges of* Q_R *in units of* mmHg/sec. *The spacing between the cathode positions is* 500 μm.

with tumour cells [10]. Haemorrhage in the tumour periphery may in addition result from leakage of blood through the open ends of new capillaries which are formed in response to an angiogenic stimulus [11].

By relating the patterns of Q_R to the histological structure of the Lewis lung carcinoma it has been possible to establish the separate Q_R values for different types of region within the tumour. The Q_R of necrotic zones was found to be appreciably less than that of viable zones but nevertheless significantly above zero (Fig. 6). There was a gradient in Q_R across larger necrotic zones and towards the edges of these zones functional respiratory enzymes and substrates were evidently retained. The necrotic zones, therefore, continued to contribute to the total tumour demand for oxygen. If radiation resistant hypoxic cells are considered to be located in zones at the boundary of necrotic regions, a non-zero Q_R of the necrotic zone would produce a gradient in oxygen tension across the hypoxic layer. This could limit the effectiveness of attempts to improve the tissue oxygenation, by for example breathing oxygen at high pressure, around points towards the venous end of the circulation where the increase in capillary oxygen tension may be small. The non-zero Q_R of necrotic zones also precludes the possibility that these zones could act as stores of oxygen which could damp out fluctuations in tissue oxygen tension produced by circulatory variations in the oxygen supply.

The changes in structure induced by irradiation of the Lewis lung carcinoma were different from those observed in the rat fibrosarcoma, SSB1a [5]. The structure of the fibrosarcoma remained unchanged for the first ten days after exposure to 2000 rad and acellularity of the tumour core only developed when the tumour started to regrow. Irradiation of the Lewis lung carcinoma resulted in the formation of a predominantly central region of necrosis during the period of growth delay when the total tumour volume remained approximately constant. The proportion of necrosis reached a maximum at about 7 days after irradiation when it was considerably greater than in untreated tumours of the same size. The changes in Q_R also differed between the two tumours. The minimum in the Q_R for viable tissue occurred at 3 days after irradiation for SSB1a and 5 days for the Lewis lung carcinoma. The Lewis lung carcinoma showed more visible evidence of damage than SSB1a with the presence of nuclear fragments and many large multinucleate cells. It was perhaps surprising that the changes in Q_R were not greater than those in SSB1a. It must be deduced that the cell fragments and abnormal cells which were found in non-necrotic regions classified with a viability index of V at 3 and 5 days after irradiation had an almost normal capacity for respiration and that a considerable proportion of the respiratory enzymes from lysed cells were retained within the tumour for several days and continued to utilise oxygen.

The marked increase in necrosis towards the centre of the irradiated Lewis lung carcinoma would seem to indicate that the tissue in the tumour core is more sensitive than that at the edge to either a direct or an indirect effect of radiation. Two possibilities can be considered: (1)—that the cells in the centre are more sensitive to the direct effect of radiation and that a certain level of killing corresponds to the formation of necrosis, (2)—an indirect mechanism, which is triggered by the local radiation damage to act at some time after the irradiation, produces necrosis of the central region.

If the first of the possible causes is considered, necrosis could result when the surviving fraction is reduced to a low level where surviving cells are too few in number to be noticed. It seems unlikely, however, that any cells could survive in the extreme necrotic conditions which are observed. A co-operative effect could be envisaged, whereby when the surviving fraction is low enough, the large number of damaged cells are toxic to the few remaining survivors so that all cells within the necrotic region eventually lyse. An increased sensitivity of central cells in a tumour is apparently contrary to the conventional view that radiation resistant hypoxic cells are preferentially distributed towards the centre. An increased radiation kill of central cells could perhaps result from a mechanism similar to that reported by Hahn [12] for Chinese Hamster cells *in vitro* where the capacity for the repair of sublethal damage was reduced for cells in stationary growth phase. However, in the Lewis lung carcinoma even hypoxic cells seem to have a considerable capacity for the repair of sub-lethal damage [13].

The second possible cause for increased necrosis in the centre of an irradiated tumour could be vascular insufficiency associated with inadequacy in the capacity for the removal of cellular debris. Although central blood vessels have generally been observed to have an intact endothelium in contrast to some

peripheral vessels [10] the spacing of the central vessels may be too great for the cellular damage to be dealt with or the blood flow may be reduced or cease altogether following the irradiation.

It is accepted that microscopic features of tumour structure on the scale of intercapillary distances play an important role in the determination of radiosensitivity particularly by influencing the number and distribution of hypoxic cells. The studies on both the fibrosarcoma, SSB1a and the Lewis lung carcinoma indicate that the responses of tumours to irradiation may also depend on larger scale features of the structure. If it is assumed that necrotic areas do not contain any viable cells, it follows, for either of the two causes of necrosis postulated above, that at the time of the formation of necrosis some cells must die in addition to those that were killed by the direct action of radiation. In addition to modifying the effect of the initial radiation exposure, the increased necrosis is likely to alter the capacity of the tumour for regrowth and the sensitivity to subsequent fractionated irradiations. The relevance of such macroscopic changes in tumour structure has often been ignored particularly in studies which have assessed the radiation response of tumours from changes in gross volume alone.

Indirect evidence for a change in the oxygen uptake of irradiated tumours has been considered in an earlier paper [5]. The present results confirm the conclusion reached then, that a change in oxygen uptake is not likely to contribute to the early reoxygenation of hypoxic zones in tumours during the first few days after irradiation. Furthermore, it is now apparent that if changes in oxygen uptake are of significance in later reoxygenation, the timing of such changes will differ for different tumour lines.

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Tumour Immunoprophylaxis Exerted by the Antimicrococcus Immunity II. Influence on the Proliferation of Murine Plasmacytoma (MOPC 173) and Ehrlich Carcinoma Cells*

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Abstract—*The influence of the micrococcal vaccine on the proliferation of normal immunocompetent and neoplastic plasma* (*MOPC* 173) *and of carcinoma* (*Ehrlich ascites*) *cells is studied.*

BALB/c mice and CDF_1 *mice, hypervaccinated with* Micrococcus Lysodeikticus, *developed significantly* ($0.001 < P < 0.005$) *lower primary immune responses against sheep erythrocytes and also displayed impaired secondary immune responses.*

Administration of Bacillus Calmette-Guérin could partially restore the immunologic potential of vaccinated mice as measured by the capability of mice to answer with primary or secondary immune responses to sheep erythrocytes but did not increase immunoresponsiveness of normal mice sensitized at nearly optimal antigenic dose.

Switching on a secondary antimicrococcus immune response after grafting 200,000 *Ehrlich carcinoma cells in the peritoneum of BALB/c mice resulted in a* 30% *increase in mean life span over control mice. However, mice hyperimmunized for months with* Micrococcus lysodeikticus *were offered the strongest immunoprotection and we found* 67% *of mice surviving to day* 90 *whereas the other vaccinated but tumour bearing animals showed a* 29% *increase in life span over control mice.*

Similarly, we demonstrated that 44% *of BALB/c mice, hyperimmunized for months, were able to reject a tumour challenge of* 200,000 *grafted MOPC* 173 *plasmacytoma cells, whereas the other micrococcus vaccinated but tumour bearing animals died with a* 26% *increase in mean survival time over control mice.*

INTRODUCTION

HYPERVACCINATION of rabbits with Gram-positive bacteria, such as *Streptococcus* [1, 2], *Pneumococcus* [3] or non-pathogenic *Micrococcus* (American Type Culture Collection 4698) stimulates the production of high titer of anticarbohydrate antibodies of restricted heterogeneity, as seen by electrophoresis (monocomponent banding immunoglobulins = M spike) and sequence analysis (homogeneous antibodies) [4]. The occurrence of those antibodies is in close analogy with the well studied [5] monoclonal immunoglobulins produced by rapidly proliferating benign plasma cells or malignant myelomas.

Antimicrococcus producing cell clones are subjected to an antigen-dependent clonal proliferation and it is common to find decreasing serum antibody levels, returning to very low levels, when vaccination is stopped.

Extensive research regarding the antibody nature of monoclonal myelomatous immunoglobulins has very clearly demonstrated, as pointed out by Waldenström [6], that "there

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is no question about the fact that anti-streptolysin and antistaphylolysin are the two most common monoclonal antibodies that are found in myeloma". But no definite role had been proposed for the true antigenic properties of those monoclonal immunoglobulins, nor is there any conclusive evidence for the role of *Streptococcus* or *Staphylococcus* in the genesis of this malignancy or in the control or maintenance of myelomatous cellular proliferation.

However, Collins and Wust clearly demonstrated that antibodies to group A *Streptococcus pyogenes* polysaccharide cross-react with tumour polysaccharides of an SV40 transformed hamster cell line and that up to 50% of the animals were offered an effective antitumour immunoprotection, when pre-vaccinated with *Streptococcus* [7].

In general oncology, it is common to find poor antibody responses and increased liability to infection associated with advanced clinical or murine cancer [8–10].

Since (multiple) myeloma growth as macroglobulinemia is often accompanied by a generalized immunosuppression [11, 12], this study was undertaken to examine if the antimicrococcus immunity, characterized by the production of oligoclonal antibody responses of high titer, may influence the expression of immune responses in vaccinated mice and if this antibacterial immunity may affect murine plasmacytoma (MOPC 173) or carcinoma (Ehrlich ascites) tumour growth.

Since antimicrococcus antibodies are directed against the bacterium specific carbohydrate (Perkins' antigen) or against the peptidoglycan [13] containing glucose and *N*-acetyl-D-glucosamine which are also specific ligands for lectins such as Concanavalin A [14] and since those antibodies bind to lymphocytes and to certain tumour cell types, but not to erythrocytes [15], they are believed to act on cells in a way similar to lectins, but with a greater discriminatory capacity.

Consequently, since lectins such as Concanavalin A are mitogenic *in vitro* [16] but were shown to be immunosuppressive *in vivo* [17], we had an additional stimulus to check the influence of the antimicrococcus immunity on immune and neoplastic cell proliferation.

The immune capacity of mice was tested by analysing the humoral immune response to sheep and horse erythrocytes injected at different stages of the immunization period. Syngeneic (inbred) mice respond to those cellular antigens with sharply resembling antibody titers of high magnitude. In contrast to the plaque forming technique, developed by Jerne, this experimental system measures the direct expression of humoral immunity and does not require animals to be killed, allowing research on the evolution of the immune response.

MATERIALS AND METHODS

Micrococcus lysodeikticus

A suspension was made of lyophilized *Micrococcus lysodeikticus* (Worthington Biochem. Corporation) after repeated washings and centrifugations at 3000 ***g*** for 15 min at 4°C, in phosphate buffered saline (0.15 M NaCl–0.01 M potassium phosphate; pH = 7.3) = PBS buffer. Micrococcus cells were heat-killed by incubating the suspension at 60°C for 30 min and used within one week.

Bacillus Calmette-Guérin

One hundred milligrams containing vials of lyophilized Bacillus Calmette-Guérin (B.C.G.) for scarification were purchased from the Pasteur Institute, Brussels. After reconstitution with the appropriate diluent and further dilution in Hanks' balanced salt solution, the suspension was kept at 4°C and used within two weeks.

Erythrocytes of sheep and horse

Ten milliliter samples of sterile, heparinized sheep or horse blood were purchased from the Pasteur Institute, Brussels and stored upon delivery in Alsever solution at 4°C.

Horse red blood cells (HoRBC) or sheep red blood cells (SRBC) were washed five times with PBS buffer and centrifuged at 300 ***g*** for 15 min. They were diluted in PBS buffer and counted in a Thoma cell haemacytometer.

Quantitative agglutination analyses

These were carried out in U shaped wells of Linbro agglutination plates. One twentieth of a milliliter of the antiserum was mixed with 0.05 ml of phosphate buffered saline and consecutive twofold dilutions were made. After a preincubation period of 30 min at 37°C, 0.05 ml of the SRBC or HoRBC (10^8 cells/ml) were added and the agglutination was scored after 2 hr at 37°C and expressed either in $\log_2$ units or by the reciprocal of the highest active dilution.

Electrophoretic procedures

Cellulose acetate electrophoreses were carried out with a Beckman microzone cell model R200.

Electrophoresis was carried out in barbital buffer (I=0.075, pH=8.6) for 20 min at 250 V at 4°C.

After staining with Ponceau S, the strips were destained with 5% acetic acid and scanned with a Vitatron Modular Photometer with scanning accessory.

Mice and tumours

Mice. Inbred (BALB/c mice × DBA/2)F_1 animals were supplied by Charles River Breeding Laboratories, Calco, Italy.

Those mice were stored at least for 3 weeks in an isolation room before we started experimenting. Female CDF_1 mice (BALB/c female × DBA/2 male)F_1 animals, weighing 19–23 g, were used. BALB/c mice were originally obtained from the T.N.O., Rijswijk, The Netherlands, fed and watered *ad libitum*. Either male mice weighing minimum 19 g or female mice weighing at least 18 g were used.

Tumours. An hyperdiploid line of the Ehrlich ascites tumour was obtained from Dr. Maisin (Belgium). This tumour has been transplanted for years by Mr. A. Cordier in inbred BALB/c mice, by weekly passage of 10^7 tumour cells to intact mice.

Ehrlich cells were aspirated from BALB/c mice and diluted in phosphate buffered saline. One tenth of a millilitre of the suspension was injected intraperitoneally (i.p.) to obtain the ascitic form of the carcinoma. Animals were randomized into test groups and control groups.

The IgG_{2a} secreting plasmacytoma 173 (MOPC 173) was kindly provided by Dr. A. Paraf (France) and weekly passaged to inbred BALB/c mice by grafting 10^6 cells to intact BALB/c mice.

Animals were randomized into test and control groups. For each experiment, ten to twenty mice were used. Mean survival time ± the standard deviation of the treated mice and the control mice (M.S.T. ± S.D.) as well as dosage and timing of each experiment are specified in the tables. Survivors on day 90 after tumour grafting were considered as long-term survivors and were eliminated from the final calculations of the mean survival time.

RESULTS

1. *Effect of hypervaccination with* Micrococcus lysodeikticus *on a primary immune response in BALB/c mice*

Previous studies in mice have demonstrated that the immune response is higher when the vaccine is injected intraperitoneally [18].

Inbred BALB/c mice were vaccinated three times a week i.p. with heat-killed *Micrococcus lysodeikticus* and received 0.05 mg per injection on weeks 1 and 2 and 0.1 mg on weeks 3 and 4, 9–14 and 19–24. They received intraperitoneally three 0.2 mg injections a week on weeks 29–31.

The capability of vaccinated mice to answer to a challenge of sheep erythrocytes was tested on weeks 16 and 33. Vaccinated and control mice were inoculated intraperitoneally with 2.10^8 SRBC and bled in the retro-orbital plexus on the fourth day. The serum anti-SRBC and antimicrococcus response was quantitatively determined by agglutination with 10^8 SRBC/ml or 2 mg/ml micrococcus suspension. As illustrated in Fig. 1, micrococcus vaccinated BALB/c mice

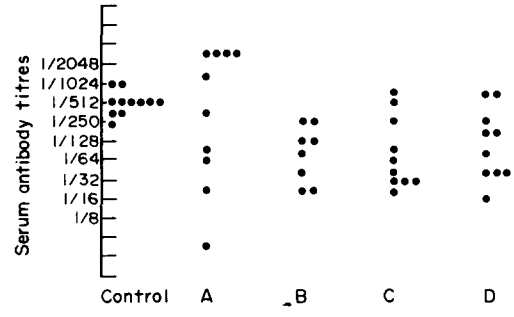

Fig. 1. Effect of hypervaccination with Micrococcus lysodeikticus *on the primary anti-SRBC response of BALB/c mice. BALB/c mice were hyperimmunized with heat-killed micrococcus vaccinated mice and control mice were determined. cinated and control mice was tested on weeks* 16 (*A*) *and* 33 (*B, C, D*) *by measuring the primary immune response to* 2.10^8 *sheep erythrocytes injected on day* 0. *Four days later, mice were bled and individual serum anti-SRBC antibody titers of micrococcus vaccinated mice and control mice were determined. Immune responses of groups B* ($P<0.001$), *C* ($P<0.001$) *and D* ($P<0.005$) *were significantly depressed.*

displayed lower anti-SRBC responses than control mice and this was more pronounced when testing on week 33 ($0.001<P<0.005$).

2. *Influence of hypervaccination on a primary response in BALB/c mice*

Intact BALB/c mice were injected with 2.10^8 SRBC on days 0, 2 and 4 and showed a strong primary immune response, as quantitatively measured by the serum agglutination titer of bleeding on day 8. Mice were randomized into test and control groups. Test group mice were vaccinated with heat-killed *Micrococcus* 3 times a week intraperitoneally and received 0.1 mg injections on weeks 1–4 and 9–12, 0.2 mg on

weeks 14–17 and 19–22 and 19–22, 0.4 mg on weeks 24–27 and 29–32 and 0.5 mg on weeks 34–37 and 39–42.

In Fig. 2, the evolution of a primary anti-SRBC response of test group and control mice bled and quantitated at different stages of the immunization period is produced.

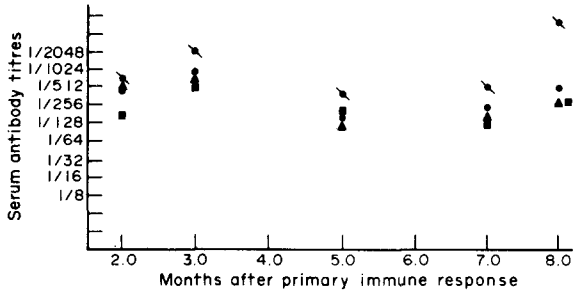

Fig. 2. Effect of hypervaccination with Micrococcus lysodeikticus *on the expression of a primary and secondary anti-SRBC response in BALB/c mice. Four groups of* 10 *BALB/c mice were primed with* 2.10^8 *SRBC and* 30 *of them were vaccinated thereafter with heat-killed* micrococcus lysodeikticus. *The expression of a primary anti-SRBC immune response is followed during hypervaccination with* Micrococcus *and compared to unvaccinated control mice (●) Eight months after priming with SRBC, Micrococcus vaccinated (▲, ■, ●) and unvaccinated control mice were boosted i.p. with* 5.10^8 *SRBC. Mean serum anti-SRBC antibody titers of this secondary immune response of vaccinated (▲, ■, ●) and control mice (●) are presented.*

Eight months after priming with SRBC, the animals were boosted i.p. with 5.10^8 cells after 44 weeks of vaccination. They were bled 4 days later and their sera were analysed for quantitative agglutinations with *Micrococcus* and SRBC. Whereas a primary anti-SRBC response switched on *before* hyperimmunization of BALB/c mice with *Micrococcus* is rather unaffected during hypervaccination, the secondary anti-SRBC response of vaccinated mice displays considerably lower anti-SRBC titers than PBS treated control mice, as illustrated in Fig. 2.

There was no direct correlation between the individual magnitude of the anti-SRBC and the antimicrococcus antibody serum titer (not shown).

3. *Effect of hyperimmunization with* Micrococcus lysodeikticus *on a primary immune response in* CDF_1 *mice*

One hundred and three intact CDF_1 mice were vaccinated once a week with 0.15 mg of heat-killed *Micrococcus* over 4 weeks. They received three 0.2 mg injections a week on weeks 10–14 and three 0.4 mg injections a week on weeks 24–29 and 31–36. Blood samples were taken from the retro-orbital plexus at different stages of the immunization period and mouse sera were subjected to cellulose acetate electrophoresis to check the presence of immunoglobulins of clonal dominance.

The serum antimicrococcus antibody titer of every bleeding was determined by quantitative agglutination with 2 mg/ml *Micrococcus* suspension. This immunization schedule resulted in the production of antibodies of restricted electrophoretic heterogeneity and clonal dominance in 5% of the mice from week 25, 26% on week 30 and 33% on week 37. Absorption of the sera with micrococcus cells results in the disappearance of electrophoretic homogeneity and precipitation with soluble cell wall antigen (immunodiffusion).

A primary anti-sheep-erythrocyte antibody response was tested after 22 weeks (before the first appearance of immunoglobulins of clonal dominance in the serum) and 36 weeks of hypervaccination. Two weeks after the last micrococcus vaccination, 2.10^8 sheep erythrocytes were inoculated intraperitoneally. As indicated in Fig. 3, CDF_1 mice vaccinated for 22 weeks and those vaccinated for 36 weeks and subdivided into non-homogeneous and homogeneous responding mice showed a depression of the primary anti-SRBC response ($P<0.001$). There was no direct correlation with the quantitative anti-micrococcus antibody titer (not shown).



Fig. 3. Influence of the antimicrococcus immunity of homogeneous and heterogeneous responder CDF_1 *mice on the primary immune response to sheep erythrocytes.* CDF_1 *mice were hyperimmunized with* Micrococcus lysodeikticus *according to an immunization scheme that leads to the production of homogeneous antibodies. Individual antibody titers of a primary anti-SRBC response of micrococcus vaccinated mice (A and B) and unvaccinated mice, tested on week* 24, *are listed.*

Antibody titers of homogeneous (C) and heterogeneous (D) responder mice, answering to SRBC with a primary immune response on week 38 *are compared with control mice.*

Immune responses of mice of groups A, B, C, and D were significantly depressed ($P<0.001$).

4. *Reversibility of the immunosuppression*

In the preceding experiment, we have tested the immunologic capacity of vaccinated CDF_1 mice, two weeks after the last injection of *Micrococcus*. This precaution was taken to insure that an immune response against sheep or horse erythrocytes should not suffer from competitive recruitment of appropriate immunocompetent and bystander cells, a phenomenon called "antigenic competition". We assumed that a rest period of two weeks is long enough for immune mice to clean up the last micrococcus cells and to recover from the injections. Since horse and sheep erythrocytes induce high humoral immune responses in mice and do not cross-react with each other, they were used as indicator systems to test the mouse immune responsiveness.

However, when the CDF_1 mice that were hyperimmunized for 36 weeks were tested after a rest period of 6 months, we found again a dramatically decreased primary anti-sheep erythrocyte immune response, even in those mice where the antimicrococcus antibody had disappeared from the serum, as seen in Table 1. Again, there was no

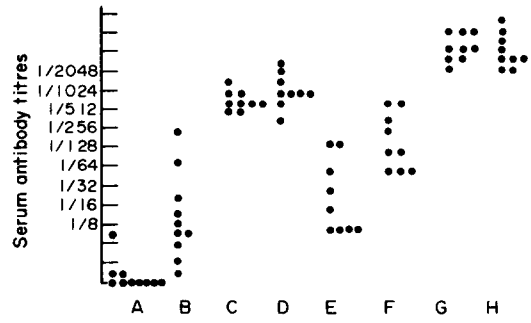

Fig. 4. Influence of B.C.G. administration on primary and secondary immune responses of micrococcus lysodeikticus hypervaccinated mice. CDF_1 mice were hypervaccinated with Micrococcus lysodeikticus *for 36 weeks, followed by a rest period of 6 months. Then, mice immunoresponsiveness was tested. Individual antibody titers of vaccinated (V^+) and unvaccinated (V^-) mice responding to 2.10^8 horse erythrocytes (H^+) with 1 mg B.C.G. (B^+) or without B.C.G. (B^-) are presented. Both primary (A–D) and secondary (E–H) immune responses are listed: A and $E = V^+$, H^+, B^-; B and $F = V^+$, H^+, B^+; C and $G = V^-$, H^+, B^+; D and $H = V^-$, H^+, B^-. Immune responses of groups A, B, E, and F were significantly depressed ($P < 0.001$).*

responses of normal mice sensitized with a near optimal antigenic dosage.

We conclude that, in micrococcus vaccinated mice that show an impaired response against new cellular antigens, the immunosuppression

Table 1. Comparison of the antimicrococcus antibody titer with the anti-SRBC antibody titer of a primary immune response in hypervaccinated mice

Micrococcus vaccinated mice						Control mice	
$\log_2$ Micro	$\log_2$ SRBC	$\log_2$ Micro	$\log_2$ SRBC	$\log_2$ Micro	$\log_2$ SRBC	$\log_2$ Micro	$\log_2$ SRBC
9	5	7	5.5	4	7	0	10
6	4.5	6	6.5	0	4.5	0	10
0	5.5	5	6.5	0	8.5	0	9.5
5	6.5	5	4.5	0	7	0	10
6	7	5	6.5	4.5	7	0	10
6	5	6	6	0	1	0	9
7	5	7	4.5	8.5	3	0	10
9	5	6	6.5	4.5	1.5	0	10

CDF_1 mice were hyperimmunized with *Micrococcus lysodeikticus* for 36 weeks, followed by a rest period of 6 months. Then, vaccinated mice and controls of the same age were injected with 2.10^8 sheep erythrocytes (SRBC) i.p. on day 0. Mice were bled on day 4 and the anti-SRBC titer of each mouse was compared to the antimicrococcus antibody titer and expressed in $\log_2$ agglutination units.

direct correlation with the quantitative anti-micrococcus antibody titer (regression $r = 0.011$). Similarly, we found that the administration of 1 mg of Bacillus Calmette-Guérin (B.C.G.) simultaneously with 2.10^8 horse erythrocytes could partially restore the immunoresponsiveness of CDF_1 mice as reflected by a primary and a secondary response anti-HoRBC response listed in Fig. 4, but was unable to amplify anti-HoRBC may be partially reversed by the administration of an additional macrophage and T cell stimulator such as B.C.G. [19].

5. *Immunoprophylaxis of the antimicrococcus immunity on the proliferation of Ehrlich carcinoma and plasmacytoma (MOPC 173) cells.*

We previously demonstrated that anti-micrococcus antibodies of rabbit and mice agglutinate Ehrlich ascites cells *in vitro* [20].

In a first experiment, BALB/c mice were immunized one injection a week (40 μg given subcutaneously) for 4 weeks. On weeks 7, 9 and 11, the animals received three injections a week.

Vaccinated mice received a tumour graft 4 months later and the randomized mouse population was divided into two groups: one group was boosted with 400 μg of heat-killed *Micrococcus* administered on days 1, 4 and 7 after tumour grafting, whereas the second group of prevaccinated mice was untreated. As illustrated in Table 2, BALB/c mice implanted (day 0) Ehrlich cells and mean survival time (M.S.T. = 29.0 days ± 8.2) of the other vaccinated but tumour bearing mice was considerably extended (M.S.T. = 22.4 dayss ± 3.1) over control mice. However, only 7% of long-term survivors were recorded 90 days after grafting i.p. 2,000,000 Ehrlich cells.

In a last experiment, we have tested the immunoprophylactic capability of an antimicrococcus immunity on the proliferation of plasmacytoma (MOPC 173) cells.

BALB/c mice were hyperimmunized with

Table 2. Effect of prevaccination with Micrococcus lysodeikticus *on murine ascites tumour growth*

Number	Tumour + dose	Micrococcus vaccinated mice Mean survival time (T) ± standard deviation (days)	Control mice Mean survival time (C) ± standard deviation (days)	T/C%	Number of cured mice vs total of micrococcus vaccinated mice
A EAT	200,000 cells	19.9 ± 2.7	20.1 ± 2.0	99	0/9 (0%)
B EAT	200,000 cells	26.1 ± 12.0	20.1 ± 2.0	130	1/9 (11%)
C EAT	200,000 cells	29.0 ± 8.25	22.4 ± 3.1	129	8/12(67%)
D EAT	2,000,000 cells	21.8 ± 8.8	19.8 ± 2.2	110	1/14(7%)
E MOPC 173	200,000 cells	13.9 ± 2.2	11.0 ± 1.5	126	4/9 (44%)

A and B: BALB/c mice immunized with one injection a week (50 μg given subcutaneously) for 4 weeks. On weeks 7, 9 and 11, animals received 3 injections a week. Vaccinated mice received a tumour graft 4 months later and some were boosted (B) with 400 μg given subcutaneously on days 1, 4 and 7 after tumour grafting.
C, D, E: BALB/c mice were vaccinated according to an immunization schedule that yields the production of homogeneous antibodies.

that had earlier shown a primary immune response did not develop any immunoprotection against 200,000 intraperitoneally implanted Ehrlich carcinoma cells. We assume that Ehrlich tumour bearing mice might be unable to recall sufficient antimicrococcus memory T cells, even in the case of shared epitopes. Micrococcus vaccinated mice, repeatedly boosted with 400 μg of heat-killed bacteria given subcutaneously on days 1, 4 and 7 after tumour grafting, displayed a significant increase in mean life span (M.S.T. = 26.1 days ± 12.0) over control mice (M.S.T. = 20.1 days ± 2.0). Then, mice were hyperimmunized i.p. for months with *Micrococcus* according to an immunization scheme described under paragraph 1 and tumour challenged on week 34.

As outlined in Table 2, BALB/c mice were given the highest immunoprotection by hypervaccination with *Micrococcus lysodeikticus:* on day 90 after tumour grafting, 67% of these mice were cured after a tumour challenge of 200,000 intraperitoneally heat-killed *Micrococcus lysodeikticus* i.p. according to the immunization scheme described under paragraph 2. On week 45, they received a tumour challenge of 200,000 plasmacytoma cells (MOPC 173) in the mouse peritoneum. As represented in Table 2, micrococcus vaccinated mice considerably resisted to a challenge of this rapidly growing neoplasm and 44% of the animals were cured on day 90, whereas the other vaccinated but tumour bearing mice displayed an increase in mean life span (M.S.T. = 13.9 days ± 2.2) over unvaccinated control mice (M.S.T. = 11.0 days ± 1.5).

DISCUSSION

In this paper, we report on the effect of hypervaccination with *Micrococcus lysodeiktikus* and its influence on the proliferation of murine immunocompetent cells and neoplastic plasma and of carcinoma cells.

By the expression of both primary and secondary humoral immune responses against sheep or horse erythrocytes, the immunologic potential of mice was measured and analysed at different stages of the immunization period. We present evidence that, depending on the immunization schedule, BALB/c mice hyperimmunized for months with *Micrococcus* displayed lower anti-SRBC responses than control mice and this is far more pronounced when mice were tested on week 33 than on week 16, as seen in Fig. 1. ($0.001 < P < 0.005$).

Figure 2 clearly shows the evolution of a primary immune anti-SRBC response during a hypervaccination schedule and demonstrates that, as compared to PBS treated animals, micrococcus vaccinated mice show an impaired secondary immune anti-SRBC response. Anti-SRBC responses of CDF_1 mice vaccinated with *Micrococcus lysodeikticus*, according to an immunization scheme that leads to the production of antibodies of restricted electrophoretic heterogeneity and clonal dominance, were dramatically depressed when tested on week 22 (before the first appearance of clonal dominant immunoglobulins in the mouse serum) and week 38 ($P < 0.001$).

Both anti-SRBC responses of homogeneous and non-homogeneous responder mice were considerably lower ($P < 0.001$) than those of control mice and we were unable to find a direct correlation with the antimicrococcus antibody titer.

This immunosuppression, generated by vaccination with heat-killed *Micrococcus lysodeikticus*, was still present after a rest period of 6 months even in those mice where the antimicrococcus antibody had disappeared from the serum (Table 1). However, we also observed that administration to those mice of 1 mg of Bacillus Calmette-Guérin together with horse erythrocytes could partially restore the immune responsiveness of vaccinated mice, as illustrated in Fig. 4. Both the bacterium and the antimicrococcus antibody may possibly account for the immunosuppression.

We previously demonstrated that rabbit antimicrococcus antisera may agglutinate lymphocytes of different species and certain tumour cell types but not erythrocytes [15], which was confirmed by others [21] who demonstrated that fluorescent rabbit anti-micrococcus antibodies induce spots and cap formation on lymphocytes of the peripheral blood and predominantly on T cells. Up to now, we ignore if this binding leads to an overstimulation, suppression or elimination of the target cell.

Alternatively, we may think that the bacterium itself may depress the immunological potential of certain lymphoid cell types in the same way as dextrans, levans [22] and pneumococcal polysaccharides [23] and that repeated administrations may induce a long-lasting reduced ability to proliferate of particular subpopulations that are required to function in the network of cellular reactions that build together an adequate immune response.

Compatible with this plausible model is the observation that CDF_1 mice hypervaccinated for five months with *Micrococcus lysodeikticus* showed an impaired anti-SRBC immunity when SRBC was inoculated intraperitoneally but were still able to build an almost normal immune response when SRBC was injected intravenously (unpublished observation).

In a third model, we may think that the antimicrococcus immunity, directed against carbohydrates such as glucose and *N*-acetyl-D-glucosamine, may selectively lead, in a way similar to lectins such as Concanavalin A, to the *in vivo* production of suppressor T cells that, whether localized or generalized, may inhibit immunologic responses [24]. However, it is worthy of note that CDF_1 mice that were hypervaccinated for months with *Micrococcus lysodeikticus* and displayed dramatically decreased immunologic responses did not have a shortened life span as compared to control mice (unpublished observation).

In a last set of experiments, we clearly demonstrated that switching on a secondary immune antimicrococcus response results in a considerable increase in mean life span of vaccinated mice over control mice bearing Ehrlich ascites tumour. When hyperimmunized for months with *Micrococcus lysodeikticus*, mice were given the strongest immunoprotection and we found 67% of cured mice on day 90 after grafting 200,000 cells, whereas the other vaccinated but tumour bearing animals showed a 29% increase in mean life span (M.S.T. = 29.0 days ± 8.2) over control mice (M.S.T. = 22.4 days ± 3.1).

The immunoprotection was severely reduced by grafting 2,000,000 Ehrlich cells to the vaccinated mice and resulted in 7% of cured mice on day 90 after tumour grafting, as outlined in Table 2. Our results show that immunosuppression occurs when

the number of grafted cells is less than 10^5 tumour cells.

Similarly, we demonstrated that BALB/c mice hyperimmunized for months with *Micrococcus lysodeikticus* were protected against a tumour challenge of 200,000 grafted plasmacytoma cells: 44% of long-term survivors were cured on day 90 and the other vaccinated but tumour bearing mice died with an increase in mean survival time (M.S.T. = 13.9 days ± 2.2) over control mice (M.S.T. = 11.0 days ± 1.5), as listed in Table 2.

With those results, we show that the antimicrococcus immunity itself obtained through hyperimmunization can be sufficient to control the proliferation of murine plasmacytoma (MOPC 173) and carcinoma (Ehrlich) cells.

This is in sharp contrast with our data obtained with the L1210 system where we clearly demonstrated [20] that booster injections were required for the antimicrococcus immunity to be operationally effective against this murine leukaemia.

Since Ehrlich ascites carcinoma (fatal tumor dose for 100% of the animals = 1000 cells) and MOPC 173 plasmacytoma (fatal tumour dose for 100% of the animals = 10,000 cells) are slightly immunogenic, whereas L1210 leukaemia is poorly immunogenic [15] (tumour dose 100% = 1–10 cells), we assume that the immunogenicity of carcinoma and plasmacytoma is sufficient to trigger enough effector cells that, in collaboration with the antimicrococcus immunity, lead to an effective immunity, whereas insufficient recruitment by the poorly immunogenic L1210 cells requires additional effector cell triggering. Those results provide additional evidence that the micrococcus vaccine might be successfully used to inhibit nascent neoplastic cell proliferation.

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Pregnancy β_1 Glycoprotein and Chorionic Gonadotrophin in the Serum of Patients with Trophoblastic and Non-Trophoblastic Tumours*

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Abstract—*Measurements of the concentration of the pregnancy associated beta$_1$ globulin, B_1SP_1, and human chorionic gonadotrophin (hCG) in sera from patients with hydatidiform mole, invasive mole, choriocarcinoma, malignant teratoma and various non-trophoblastic tumours have been made in a multi-centre study by radioimmunoassay. Estimations of B_1SP_1 and hCG in different laboratories correlated to a degree consistent with the conditions of the study. The correlation between B_1SP_1 and hCG values in patients with gestational trophoblastic tumours was not close and that in patients with malignant teratomas was poor. Values for hCG were usually higher than those for B_1SP_1, suggesting that, in general, hCG is likely to be the more useful and consistent marker. HCG values* > 3000 ng/ml *were always associated with detectable levels of B_1SP_1. The possibility that B_1SP_1 measurements have a place in detecting minimal residual disease states, has not, however, been excluded by the present study. Elevated values of B_1SP_1 were not found in a small group of patients with non-trophoblastic tumours.*

INTRODUCTION

A PREGNANCY associated beta-globulin has been identified in the sera of pregnant women by an antiserum against pregnancy [1, 2] and against a protein fraction of placenta [3]. It ia referred to here as B_1SP_1. The biological function of this glycoprotein has not yet been defined but it has been localised in both normal and malignant trophoblastic cells [4–6] and in some non-trophoblastic tumours [7]. Oestrogen-binding [8] and immunosuppressive properties [9] have been described.

Trophoblast synthesizes several polypeptides and one of these, human chorionic gonadotrophin (hCG) is widely used as the basis of pregnancy tests. Quantitative estimations of this hormone are used to monitor hydatidiform mole, invasive mole, choriocarcinoma and trophoblast-like elements found in malignant teratomas [10]. Two other trophoblastic polypeptides, human placental lactogen (hPL) and placental alkaline phosphatase (PAP) have been identified in the sera of some patients with trophoblastic tumours but as markers for trophoblastic tumours they have proved inferior to hCG. It is relevant that both hPL and PAP are characteristic of the trophoblast of late pregnancy whereas hCG and B_1SP_1 are both produced in relatively large amounts by early pregnancy trophoblast [11–13]. Since hCG shares some antigenic determinants with pituitary luteinizing hormone (LH), assay specificity depends on using antisera directed at the B sub-unit of hCG ([14]. B_1Sp_1 is not known to have an analogue in the normal non-pregnant adult and it therefore offers the potential of more specific detection of minimal residual tumour.

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In the present study we have made a preliminary comparison of B_1SP_1 and hCG as markers for trophoblastic and some non-trophoblastic tumours.

MATERIALS AND METHODS

Four laboratories participated in the study and for the present report are designated A, B, C, D. Samples of sera were collected in the hospitals associated with the participating laboratories and stored at −20°C until transported in the frozen state to the W.H.O. laboratory at Lyons. The samples were then thawed, aliquoted, coded and returned frozen to the participating laboratories. The sources of sera, on which both B_1SP_1 and hCG measurements were made, are shown in Table 1.

Table 1.

Gestational trophoblastic tumours*	83
Malignant teratoma†	23
Non-trophoblastic tumours‡	23
Normal female blood-donors	20
Positive controls (late pregnancy sera)	11
	160

*Includes hydatidiform mole, invasive mole, choriocarcinoma.
†Includes embryonal carcinoma, teratocarcinoma, seminoma.
‡Includes fibromyoma and carcinomas of cervix and endometrium.

Estimations of β_1SP_1 were made in laboratories A & B; estimations of hCG by β-subunit assay were made in laboratories C & D. The estimations reported here were all made by radioimmunoassays but no attempt was made to use common reagents or reference materials or to standardise methods.

Laboratory A used Behringwerke Standard β_1SP_1 for reference standards and for isotopic labelling. The anti-β_1SP_1 serum was raised in rabbits and a 2-stage incubation system was employed.

Laboratory B prepared its own β_1SP_1 from pooled pregnancy sera and compared this with Behringwerke Standard. It used a rabbit anti-β_1SP_1 with a 2-stage incubation in the second antibody precipitation method. Both laboratory A and B expressed results as ng/ml.

Laboratory C and laboratory D used their own laboratory purified preparations of hCG prepared from pregnancy urine sources for isotopic labelling with ^{125}I and as laboratory standards.

Laboratory C expressed its results in weight of laboratory standard and laboratory D in terms of the 2nd International Reference Preparation of hCG. Both laboratories C and D used double antibody methods but laboratory C employed a 2-stage incubation whereas laboratory D used a one stage incubation with pre-precipitated antisera, the first antisera in each case being of rabbit origin.

The amount of sera available did not allow all laboratories to obtain results on all the specimens but at least one result for each polypeptide was obtained on the samples reported here. The effect of freezing and thawing the samples on estimations in the participating laboratories was not studied.

RESULTS

β_1SP_1

The limit of sensitivity in laboratories A and B were 3 ng/ml and 2 ng/ml respectively. Observed values ranged from the limit of sensitivity up to 1500 ng/ml and 37,000 ng/ml respectively. The mean values for all specimens examined was 109 ng/ml in laboratory A and 49 ng/ml in laboratory B. The coefficient of correlation between these laboratories was 0.919 (paired T value 1.62 for 59 degrees of freedom).

The mean values for patients with gestational trophoblastic lesions in the two laboratories were 309 and 138 ng/ml (correlation coefficient 0.914) and for malignant teratomas the mean values were 37 and 16 ng/ml (correlation coefficient 0.916). The mean values for normal female blood donors were <4 ng/ml. For patients with non-trophoblastic tumours the mean values did not differ significantly from the normal controls <4 ng/ml.

hCG

The lower limits of the working range in the laboratories for this study were 4 ng/ml in laboratory C and 10 m.i.u. in laboratory D. Values observed ranged from the lower limit of the working range to 410,000 ng/ml and 560,000 mi.u./ml respectively. Mean values were 17,495 ng/ml and 51,565 mi.u./ml The coefficient of correlation between the laboratories was 0.693 (paired T value −2.32 for 76 degrees of freedom). The mean values for patients with gestational trophoblastic

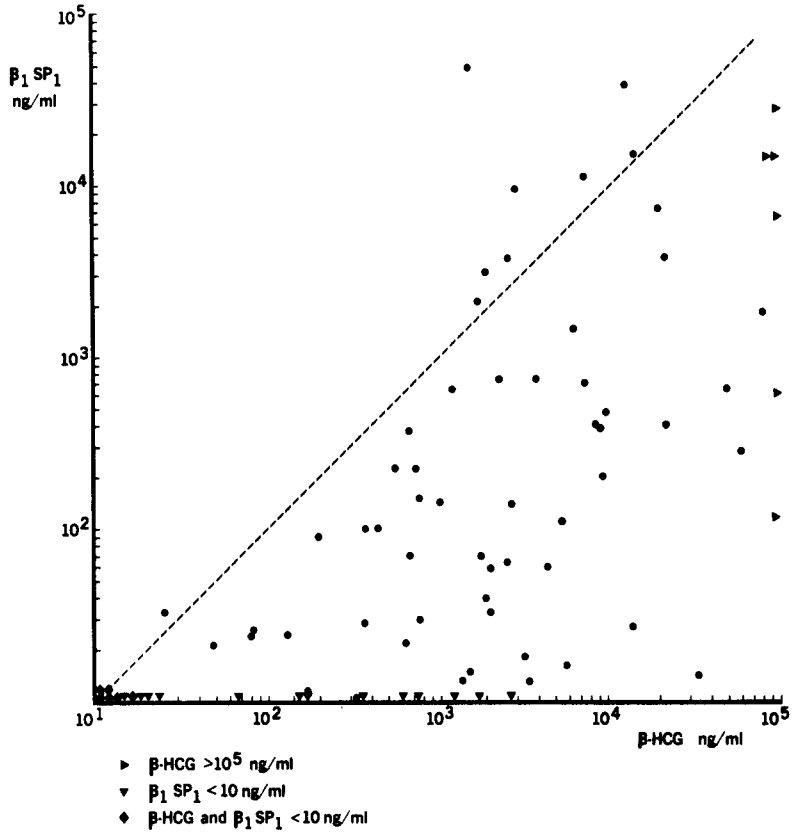

Fig. 1. Relationship between serum β-HCG and $\beta_1 SP_1$ in patients with gestational trophoblastic tumours.

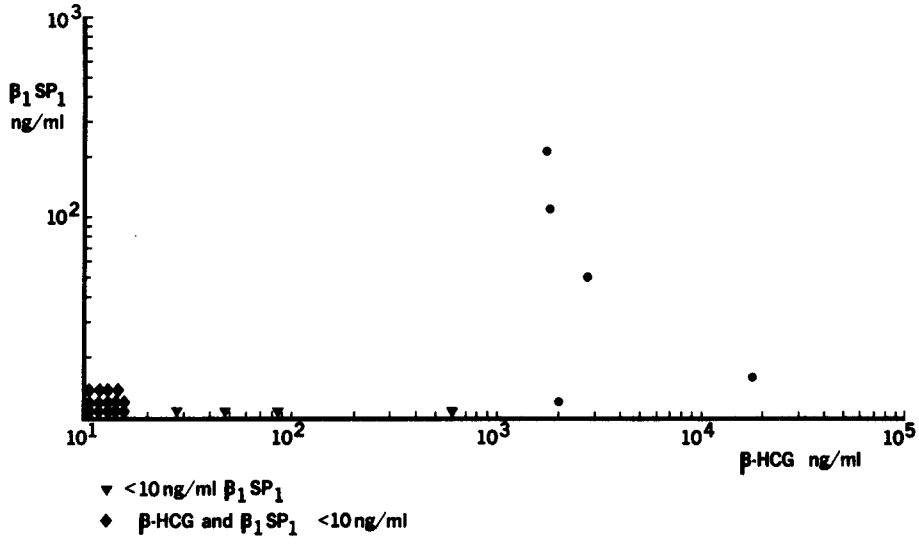

Fig. 2. Relationship between serum β-HCG and $\beta_1 SP_1$ in patients with teratocarcinoma and other gonadal tumours.

tumours were 40,583 ng/ml and 119,384 mi.u./ml (correlation coefficient 0.651) and for malignant teratomas were 415 ng/ml and 119. mi.u./ml (correlation coefficient 0.993). For normal blood donors the means were <4 ng/ml and <10 mi.u./ml respectively. For non-trophoblastic tumour patients the mean values did not differ significantly from the normal controls in laboratory D but were marginally higher in laboratory C.

Correlation between $\beta_1 SP_1$ and hCG

The mean ratio hCG/B_1SP_1 for all samples was 4.3/1.0; for gestational tumours it was 14.6/1.0 and for malignant teratoma it was 113.3/1.0. The correlation between estimated concentrations of the two substances in the same specimens (using the data from laboratories B and C only) in gestational trophoblastic tumours was 0.281 and for malignant teratomas was 0.164 (see Figs. 1 and 2).

In the group with gestational tumours there were 2 samples where hCG was not detected in either laboratory but low levels of B_1SP_1 (3–10 ng/ml) were detected. There were 2 samples where B_1SP_1 was not detected but low levels of hCG were recorded. In 9 instances the concentration of B_1SP_1 exceeded the concentrations of hCG but in 63 instances hCG concentration exceeded B_1SP_1 (Fig. 1).

Estimates of the two substances in the sera of patients with malignant teratoma gave widely differing correlation coefficients; for laboratories B and C the coefficient was 0.164 (22 degrees of freedom) and for laboratories B and D it was 0.657 (14 degrees of freedom). In no cases did the B_1SP_1 value exceed the hCG concentration and in no instance was B_1SP_1 detected in the absence of hCG. In 4 cases hCG was detected in the absence of B_1SP_1 (Fig. 2).

DISCUSSION

It is well known that when assays are performed in different laboratories using different assay techniques, reagents and standards, wide variations are likely in the numerical values observed and that the correlation between results may be poor. Such studies may however reveal broad patterns of agreement. The correlation between estimates in the B_1SP_1 laboratories in the study was satisfactory but that between the hCG laboratories was less so except in the case of malignant teratomas where the correlation was good.

The present data indicate that hCG values are generally higher than B_1SP_1 values in the sera of patients with active gestational trophoblastic tumours and malignant teratomas. The metabolic half-lives of these substances are probably similar, that is about 30–50 hr, [15, 16], so the differences are likely to reflect different rates of synthesis and secretion. The values of both hCG and B_1SP_1 in the sample of patients with non-trophoblastic tumours revealed no significant elevations above control values but the sample was small and firm conclusions would be unwarranted.

Although hCG values were generally higher than those of B_1SP_1, suggesting that hCG is likely to remain the most useful marker for choriocarcinoma, there were, nevertheless, few samples where B_1SP_1 was undetectable and in 2 instances B_1SP_1 was detected but hCG was not. In order to determine whether B_1SP_1 measurements might, for instance, reveal minimal residual disease not detected by hCG measurements further studies will be required. It is also necessary to determine whether the relationships of the two substances have prognostic implications. There were no instances where a high level of one marker was associated with undetectable levels of the other although B_1SP_1 values <10 ng/ml were sometimes found with hCG values in the range of 50–5000 ng/ml. It seems clear that although B_1SP_1 and hCG are probably synthesized by the same cells dissociation in their respective rates of synthesis or metabolism takes place.

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Oestrogen Receptors and Peripheral Serum Levels of Oestradiol-17β in Patients with Mammary Carcinoma*

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Abstract—*Oestradiol receptors were determined by isoelectric focusing in cytosol from breast cancer tissue from* 12 *pre- and* 70 *postmenopausal patients. Simultaneous determination of the peripheral serum levels of oestradiol-*17β *was carried out in the* 12 *premenopausal and* 57 *of the postmenopausal patients. At oestradiol-*17β *levels below* 150 pM *the receptor values ranged from below* 0.01 *to* 7.19 fmole *bound* [^{3}H] *oestradiol-*17β/μg *of DNA. At oestradiol-*17β *levels above* 150 *pM only low receptor values* (<0.25 fmole *bound* [^{3}H] *oestradiol-*17β/μg *of DNA) were recorded. The results indicate an influence of the endogenous oestrogen on the receptor assay probably due to blockade of the hormone binding site. It is proposed that an endocrine evaluation of the patient should be performed together with the oestrogen receptor assay.*

INTRODUCTION

THE DETERMINATION of oestrogen receptors in breast cancer tissue can now be considered as an established tool for the selection of therapeutic regimens [1, 2]. Forty to sixty % of the receptor positive tumours are reported to respond favourably to different kinds of endocrine therapy, while the corresponding figure for receptor negative tumours is about 5% [1]. It is therefore important to know the factors influencing the receptor assay. It has been reported that oestrogen receptor positive tumours are more frequent among postmenopausal patients [1] and it is well known that the production and peripheral levels of oestrogens decrease dramatically during the menopause. This indicates an effect of endogenous oestrogen on the results of the receptor assay. Furthermore, a relation between high plasma levels of oestradiol-17β and low binding of [^{3}H]-oestradiol-17β in slices of human breast cancer tissue has been reported by Maass *et al.* [3] although very few patients with high oestrogen levels were included in that study. Sakai and Saez [4] have recently reported a positive correlation between high plasma levels of oestrogens and the degree of saturation of the receptor in breast cancer tissue.

In order to further investigate the influence of endogenous oestrogen upon the results of oestrogen receptor assays and endocrine treatment, we have determined free oestrogen receptors in breast cancer tissue from pre- and postmenopausal patients using a newly developed technique based on isoelectric focusing [5]. The receptor values have been correlated to the patient's age and the peripheral serum levels of oestradiol-17β.

MATERIALS AND METHODS

Clinical material

The clinical material consisted of 82 patients with histologically confirmed breast cancer. Twelve of the subjects were premenopausal and 70 postmenopausal. Oestrogen receptor analysis was performed in all patients and peripheral serum levels of oestradiol-17β were determined in the 12 premenopausal subjects and in 57 of the postmenopausal subjects.

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Determination of oestrogen receptors

Surgical biopsies were performed and immediately transferred to the laboratory in ice cold saline. They were trimmed free from fat and normal tissue and analyzed immediately or frozen at −70°C. Fresh or rapidly thawed carcinomas were mixed and suspended in three volumes of ET-buffer (10 mM Tris–HCl pH 7.4; 1.5 nM disodium-EDTA) containing 7.5 nM [^{3}H]oestradiol-17β at 0°C for 30 min. After that the mince was homogenized, further incubated at 0°C for 45 min and centrifugated. The supernatant was subjected to limited trypsin digestion at 10°C for 30 min and the receptor content was determined by isoelectric focusing in slabs of polycrylamide gel. The procedure summarized above is described in detail in a previous communication [5]. The DNA content in the homogenate was determined according to Burton [6] and the results were expressed as fmole bound [^{3}H]oestradiol-17β per μg of DNA.

Determination of serum oestradiol-17β

The determination of oestradiol-17β in peripheral serum was performed after ether extraction by radioimmunoassay using anti-oestradiol-17β-6 (carboxymethyl oxime) bovine serum albumin. This antibody cross reacts to 11% with oestrone [7].

RESULTS

According to a previous study from this group [8] tumours with receptor values less than 0.2 fmole/μg DNA were not associated with clinically significant response to tamoxifen therapy and thus regarded as negative in this respect. Of the 82 patients studied 25 had oestrogen receptor values less than 0.2 fmole/μg DNA. Ten of the twelve pre-menopausal subjects were included in this group. The age of all patients ranged from 26 to 83 yr and the oestrogen receptor values are listed according to the patient's age in Fig. 1. In Fig. 2 the oestrogen receptor values are listed according to the peripheral serum levels of oestradiol-17β. Oestradiol-17β-values exceeding 150 pM are only associated with oestrogen receptor values less than 0.25 fmole/μg DNA.

DISCUSSION

The results of the present investigation confirm the tendency to low binding of [^{3}H]oestradiol-17β at high peripheral

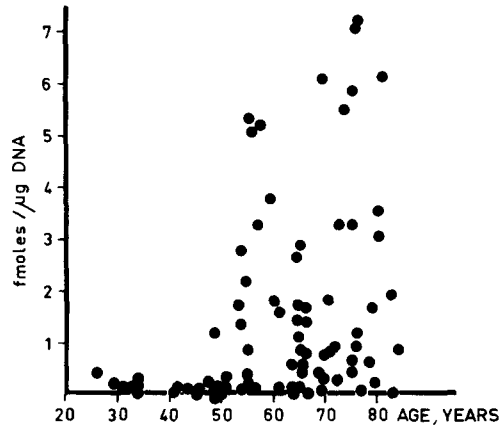

Fig. 1. Oestrogen receptor values in breast cancer tissues listed according to the patients age.

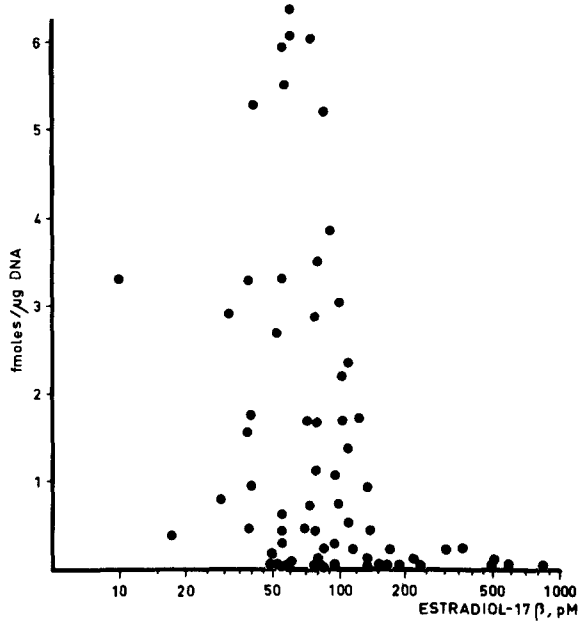

Fig. 2. Oestrogen receptor values in breast cancer tissues listed according to serum oestradiol-17β.

oestrogen values shown by Maass *et al.* [3]. This is not unexpected since a fraction of the receptors occupied by endogenous hormone will be located in the nuclei. Similar results can also be extracted upon re-examination of the data presented by Sakai and Saez [4]. When they plotted their receptor values against the plasma levels of oestrone + oestradiol-17β no clear tendency was found in this respect. If one, however, plots their receptor values against the levels of oestradiol-17β only, a clear tendency to low receptor values at high oestradiol-17β levels (above 150 pM) can be demonstrated. It should be pointed out

that the plasma levels of unconjugated oestrone presented by Sakai and Saez and the oestrone/oestradiol-ratios which can be calculated from their data by far exceed the corresponding figures given by other authors for healthy subjects as well as for breast cancer patients [9–13]. Methodological factors might therefore explain the lack of tendency when the plasma levels of oestrone + oestradiol-17β was used by Sakai and Saez as an endocrine parameter.

Fishman *et al.* have recently studied the relationship between cytosol oestradiol-17β receptors and the oestrogen content in human breast cancer tissue [14]. They claimed no relation between high oestrogen content and low receptor measurements. However, no data concerning their patients were presented. It is well known that women with low peripheral oestrogen levels can show high as well as low receptor values (Fig. 2). This wide range of receptor values may reflect differences between tumour cells with regard to receptor levels and/or cellular heterogenity of the tumours, i.e. different amounts of receptor-containing cells in different tumours [2]. Another reason may be differences in the oestrogen metabolism in the tumours, especially in the formation of oestradiol-17β from other oestrogens [4]. Such metabolic differences may, however, be of less significance at higher oestrogen levels due to the large amounts of available oestradiol-17β. Garola and McGuire [15] have recently shown that increased peripheral levels of oestradiol-17β due to exogenous administration caused a pronounced decrease in the cytosol receptor values in DMBA induced mammary tumours in the rat. Concomitantly a corresponding increase in the nuclear receptor values was observed. This experiment clearly demonstrates the influence of peripheral oestrogen levels upon the results of the cytosol receptor assay.

From the present results it can be concluded that an endocrine evaluation of the patient should be carried out together with the receptor assays. Exchange studies and assay of the nuclear receptor may be of value at least in premenopausal patients. In the endocrine evaluation the oestrogen metabolic pattern in the tumour tissue may give information of importance for evaluation of the receptor assay. Studies on this topic are in progress in our laboratories and the results will be published elsewhere.

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"Spontaneous" Neoplastic Transformation *In Vitro* of Epithelial Cell Strains of Rat Liver: Cytology, Growth and Enzymatic Activities

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Abstract—*We can follow in culture the spontaneous neoplastic transformation of one epithelial cell strain derived from normal rat liver and determine, by comparison with other liver cell strains, the divers modifications which appear before or during transformation.*

A decrease in adhesiveness seems to be one of the first manifestation. Increase in saturation density, ability to grow in soft agar and acquisition of tumorigenicity appear much later.

Moreover, in contrast to the other cell strains, in this transformed cell strain, some enzymatic activities were increased and tyrosine aminotransferase was induced by dexamethasone.

INTRODUCTION

THE DEVELOPMENT of the hepatic cell culture system is important in the assay of potentially carcinogenic drugs and for studies on the mechanism of cell transformation. Since the report by Berry and Friend on differentiated normal hepatocytes purified after perfusion with collagenase [1], a number of investigators have shown that isolated parenchymal cells do not proliferate *in vitro* and do not give rise to clones of liver epithelial cells [2–4]. By contrast, some authors have obtained epithelial cell strains from normal liver by other procedures [5–9]. Generally, these cells do not closely resemble hepatocytes, being structurally simpler, they appear to stem from hepatic cell types that proliferate quickly in culture. These cells, often fail to show hepatocyte-like biochemical properties [10] such as tyrosine aminotransferase (TAT) which have been chosen as a hepatic marker for its response to glucocorticoids. On the other hand, adult liver contains specific "markers" such as the isozymes aldolase B and pyruvate kinase L. Isozymes are multiple molecular forms, genetically determined; they may have different properties, but they have the same action on the principal substrates and seem to represent a high degree of differentiation. In several pathological conditions, especially in neoplastic tissues, and in primary hepatocyte cultures, modifications of the liver isozymic pattern have been reported [11–17].

Moreover, certain proteins have been found in normal liver of different animals which inhibit the cell growth of cultures [15] and tissues, especially adult liver growth after a partial hepatectomy [16]. These authors report this "inhibitory factor" as specific to liver tissue and as being present in a small amount in hepatoma [15]. Its presence has not yet been investigated in hepatic cell strains.

Malignant conversion *in vitro* of cultured liver cells has often been induced or accelerated by treatment with chemical carcinogens or oncovirus but few reports concern spontaneous transformations of liver cells *in vitro*. The detection of malignant conversion *in vitro* of epithelial cells is more difficult than for fibroblasts. This is because little alteration appears in the cell morphology. However growth and functional changes and neoplastic properties can also occur during transformation. Since a number of variations from

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one clone to another have been reported with the same line, and from one cell line to another in different induced malignant transformations *in vitro*, it is interesting to analyze the gradual changes which occur during "spontaneous" transformation.

The purpose of the present paper is

(a) to describe the principal cytological, growth and functional changes which gradually appear in different isolated epithelial cell strains derived from normal livers when they spontaneously transform in culture;

(b) to compare the changes of each parameter at sequential passages;

(c) to compare their new characteristics to those of a hepatoma cell strain.

MATERIALS AND METHODS

I. *Cell strains and culture conditions*

The cell strains F_I, F_{II}, F_{III} were initiated from normal liver of 10-day old Fischer or Commentary rats. The Williams' procedure was employed using 0.25% trypsin and the sequential passage of primary cultures to achieve an elimination of fibroblasts [8]. The cells were grown in Williams' medium supplemented with 15% fetal bovine serum, glutamine (0.293 g/l) and antibiotics (neomycin 0.05 g/l, streptomycin 0.05 g/l, penicillin 200,000 U/l).

Hepatoma cells LF derived from transplantable tumour. This tumour originated from a hepatoma induced in Commentry rats by a carcinogen, dimethylaminoazobenzene (DAB). The cell strain was initiated by consecutive dissocations with 0.25% trypsin solution. The cells were grown in MEM medium with 20% bovine serum (56°C heat inactivation) and supplemented with glutamine and antibiotics as above. The main difference between the two media is that Williams' medium contains more aminoacids, vitamins and ions (Zn, Cu, Fe, Mn) than MEM and that Williams' medium is supplemented with fetal calf serum instead of calf serum.

II. *Preparation of samples for assays*

To investigate the "inhibitory factor" in the cell strains, monolayers of cells were washed 3 times with PBS and scraped from the dishes with a rubber policeman, suspended in PBS and counted with the coultronic Coulter (F'). The cell suspensions were lysed by sonication for 45 sec and the lysates were centrifuged at 5000 rev./min for 10 min. The supernatant was filtered on a 0.45 μ Millipore® filter.

For the enzyme studies, we used a cell extract supernatant obtained by detaching the cells with 0.25% trypsin solution, washing them in PBS, followed by freezing and thawing 3 times and centrifuging at 12,000 rev/min for 10 min.

Morphological changes were observed by light and electron microscopy using routine preparation methods.

Neoplastic transformation was demonstrated *in vitro* by the growth of colonies in soft agar [17]. It was confirmed by tumour production *in vivo* after intraperitoneal injection of about 2×10^6 cells into syngeneic newborn rats.

III. *Enzyme and protein assays*

Enzymes were assayed by the following methods: aldolase, Sibley and Lehninger's [18]; pyruvate kinase, Bücher and Pfleiderer's [19]; lactate dehydrogenase, Wroblevski and Ladue's [20]; glucose-6-phosphate dehydrogenase, Beutler's [21]; tyrosine aminotransferase, Granner and Tomkin's [22]. Enzymatic activities were expressed in International units (i.u.) per g of protein. Total proteins were measured by the Lowry procedure [23].

For the electrophoretic studies, the pyruvate kinase isozymic pattern was resolved by isoelectrofocusing using Vesterberg's method.[24] with some modifications [25]. Aldolase isozymes were separated by the electrophoretic method of Penhoët *et al.* adapted to starch gel [26].

The "inhibitory activity" of the cell extracts was defined by their effect at different dilutions on the cell growth of the hepatoma cell strain LF, during 24 hr of incubation. Results are expressed in per cent of growth inhibition compared to the controls.

RESULTS

I. *Growth and morphological characteristics of the hepatic epithelial cell strains during the first passages*

Many cell strains have been successfully established in our laboratory using successive trypsination. Three of them shown in Table I were studied: F_I and F_{II} were initiated from Fischer rats, and F_{III} from Commentry rats. They divided slowly and reached confluency several weeks after seeding. Thereafter, for subcultures, when 10^5

Table 1. *Rat liver and hepatoma cells cultures*

Cell strains >30th passage	Origin	Rat strain	Morphology	Plating* efficiency (average) %	Colony† forming cells in soft-agar average %	Tumor production‡ No. rats with tumors / No. inoculated rats
F_I	Normal liver	Fisher	Epithelial	27	0.16	12/15
F_{II}	Normal liver	Fisher	Epithelial	2	0	0/10
F_{III}	Normal liver	Commentry	Epithelial	29	0	0/20
LF	Hepatoma	Commentry	Epithelial	51	0.23	18/20

*0.25–1 × 10^3 cells seeded/plate (average 5–10 plates).
†5 × 10^4 cells seeded/plate (average 3 plates—colonies counted 12 days after plating).
‡2–3 × 10^6 cells inoculated (0.2 ml i.p./newborn rat).

cells/ml were seeded in 6 cm^2 dishes, confluency was reached after 4–5 days of culture.

During the first passages, their mode of growth was a flattened epithelial shape and cells had a very high adhesion to the substrate on which they grew and to their neighboring cells (Fig. 1). Tight junctions between the cells were seen by ultrastructural studies.

II. *Spontaneously-transformed liver cell strains and hepatoma in culture: growth and morphological characteristics*

In contrast to the F_{II} and F_{III} cell strains which still conserve some normal characteristics, one of the strains studied F_I (Table 1) on the 30th passage, in normal conditions, appeared to present some evidence of neoplastic transformation.

F_I cells gave rise to colonies in soft-agar similar to the hepatoma strain LF. In addition, intraperitoneal injection of 2–3 × 10^6 cells into syngeneic newborn rats gave rise to carcinomas in 80% of the animals at the different stages studied: 31st, 37th, 48th passages. However, the development of tumours was always very slow (4–9 months) in comparison to hepatoma cells which needed, in the same conditions, 10 days to produce tumours at each passage studied (18th to 500th). The histological studies of the nodular granulations obtained with F_I cells showed some aspects of tumour cells from little-differentiated hepatoma. By contrast, injection of LF hepatoma gave rise to tumors with the characteristic trabecular morphology of hepatoma. Using ultrastructural studies, no evidence of viral and PPLO particles was observed in F_I, F_{II}, F_{III} and LF cells.

One of the first manifestations of the transformation of F_I cells was a decrease of their high adhesion: it was progressively easier to detach the cells from their substrate by trypsin action through the 18th to the 30th passage. Gradual overlapping in the culture also occurred very early (near the 13th passage), but the cells remained epithelioid, and never piling up as LF hepatoma cells (Fig. 2). In both these strains, F_I and LF, there was a marked decrease in mutual cell contact. These cells had many prominent surface microvilli and no tight-junctions were observed by microscopic studies.

Increase of the protein/cell ratio by a factor of 3 in the cell strain F_I was the same as in the hepatoma LF. This ratio remained unchanged in F_{II} and F_{III} cells and.these cell strains still conserved an apparent normal aspect.

Nevertheless, in comparison to F_{II} cells which did not change, the adhesiveness of F_I and F_{III} cells decreased progressively. In addition, the generation time appeared strongly reduced and the saturation density was increased by a factor 4 for F_I on the 132nd passage and by a factor 3 for F_{III} on the 66th passage. However they had not yet reached the high saturation density of LF hepatoma cells, which increased by a factor 8 on the 91st passage. Confluency was complete earlier for F_I and LF (after 2–3 days of culture instead of 4–5 days for F_{III}).

However, in spite of the transformation shown above for F_{III}, these cells never produced colonies in soft-agar even on the 65th passage and did not give rise to tumor in rats after intraperitoneal injection (43rd

passage). It was the same for the F_{II} cell strain studied which are still on the 40th passage. But, we have observed that using Williams' medium instead of MEM medium even the neoplastic LF hepatoma cell strain fails to grow in soft agar.

Thus, if the F_I cell strain shows evidence of spontaneous neoplastic transformation, the F_{III} cells strain appears to be in the process of transforming. For F_{II}, which still keeps some of the normal aspect of liver cells, we think that it could be transformed later like the others.

The isozymic pattern was the same in all the cell strains studied. Pyruvate kinase L (liver type) was absent and the K type (kidney and fetal type) was the only one seen during the cell growth and at confluency. Electrofocusing showed that the two subbands of the K form, M_2 and M_4 were present, M_2 being the most predominant (Fig. 3). For aldolase, hybrids ac were found (A is the muscle and fetal type; C is found in brain and in fetal liver). The specific form of adult liver, B type, was never seen in any of the strains studied (Fig. 4).

Table 2. Enzymic activities (in i.u./g *of protein) of normal and transformed cells*

	Normal hepatocytes	Epithelial cells F_{III}	Epithelial cells F_{II}	"Spontaneously" transformed epithelial cells F_I	Hepatoma cells LF
Pyruvate kinase	120	185	402	770	625
Aldolase	19	8.5	17	23	127
Lactico-dehydrogenase	2590	690	643	772	7800
Glucose-6-phosphate Dehydrogenase	60	66	240	270	560
Tyrosine amino-transferase	15	0.25	0.3	0.3	0.5

III. *Enzyme activities and specific characteristics of liver-cell strain during their spontaneous transformation*

The activity of pyruvate kinase, aldolase, glucose-6-phosphate dehydrogenase, shown in Table 2, appeared to be increased in the spontaneously transformed cells F_I in comparison to F_{II} and no evident increase was observed for F_{III}. The lacticodehydrogenase activity was very high in all cases, especially in LF hepatoma cells.

Induction of TAT by adding 10^{-5}M dexamethasone to the F_I cell medium was observed after 18 hr of action. Specific activity of TAT, was increased 2–5 times in the F_I cell strain. By contrast, no TAT induction was observed with F_{II} and F_{III} strains.

Table 3 shows that different concentrations of the F_I cell-strain extracts during its 16 first passages, inhibited the cell growth as did extracts of rat liver. But no inhibitory effect was observed after the 34th passage

Table 3. Inhibition of cell growth after 24 hr *of action of adult rat liver extracts and of cell strain F_I extracts at different passages*

Concentration of extracts* —in mg of liver/ml medium for liver extracts —in 10^6 cells/ml medium for cell extracts	Inhibition of cell growth in culture in % by extracts of: Adult rat liver	Cell strain F_I at different passages 15th	16th	34th	45th	116th
5	63	—	—	0	—	0
2	60	50	47	0	0	0
1	51	35	—	0	0	0
0.2	35	19	—	0	—	0

*1 mg of liver of 10^6 cells correspond approximately to 50 μg proteins.
It is expressed in % of the control growth,
(0) expresses the absence of inhibition.
(—) expresses the absence of experiment.
Mean of 2–4 experiments.

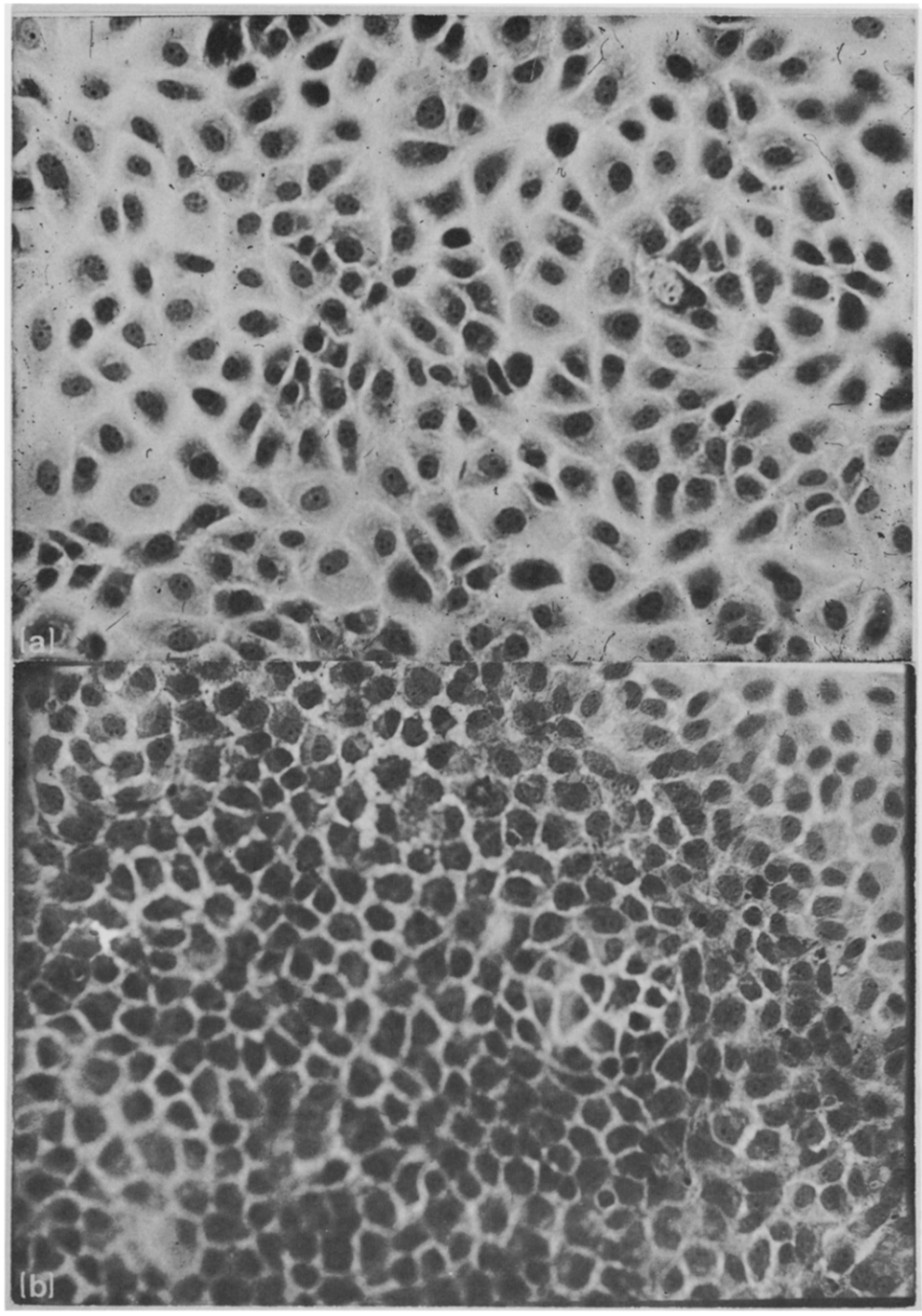


Fig. 1. F_1 *rat liver cells in culture* (a) 4th *passage. Mosaic like monolayer of non overlapping cells* (× 175). (b) 32nd *passage. Spontaneous neoplastic transformed line with some overlapping* (× 175).

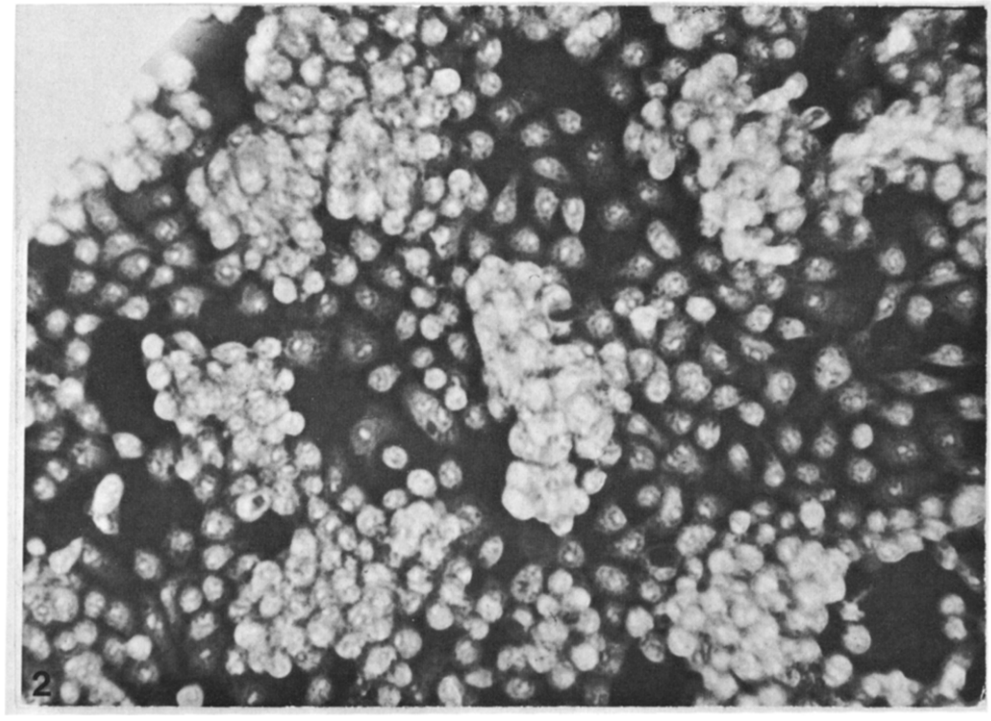
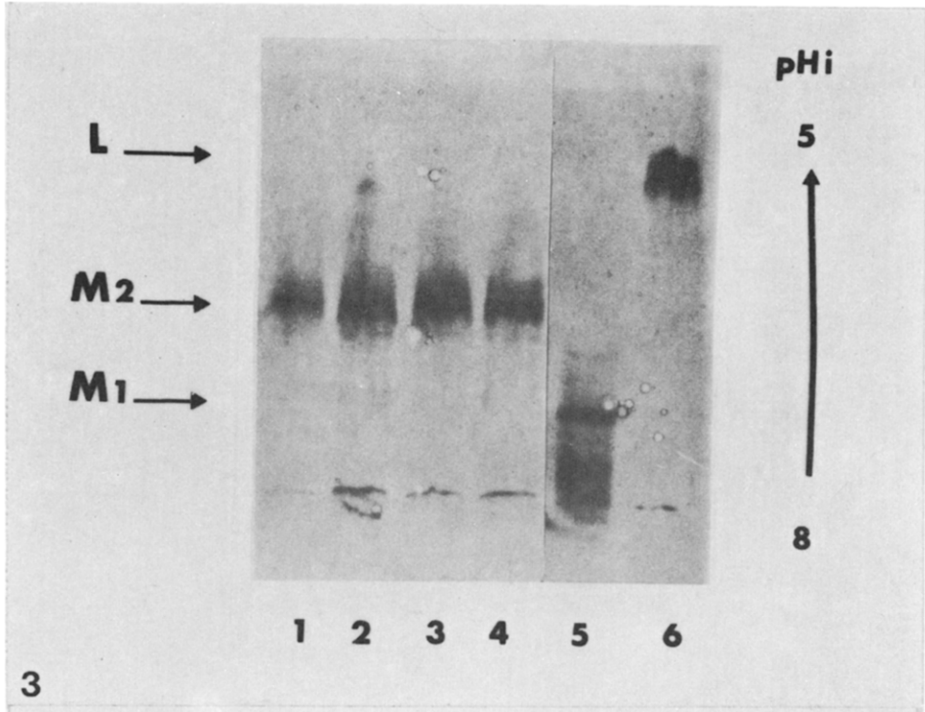


Fig. 2. LF: Rat hepatoma cells in culture showing piling up (×175).



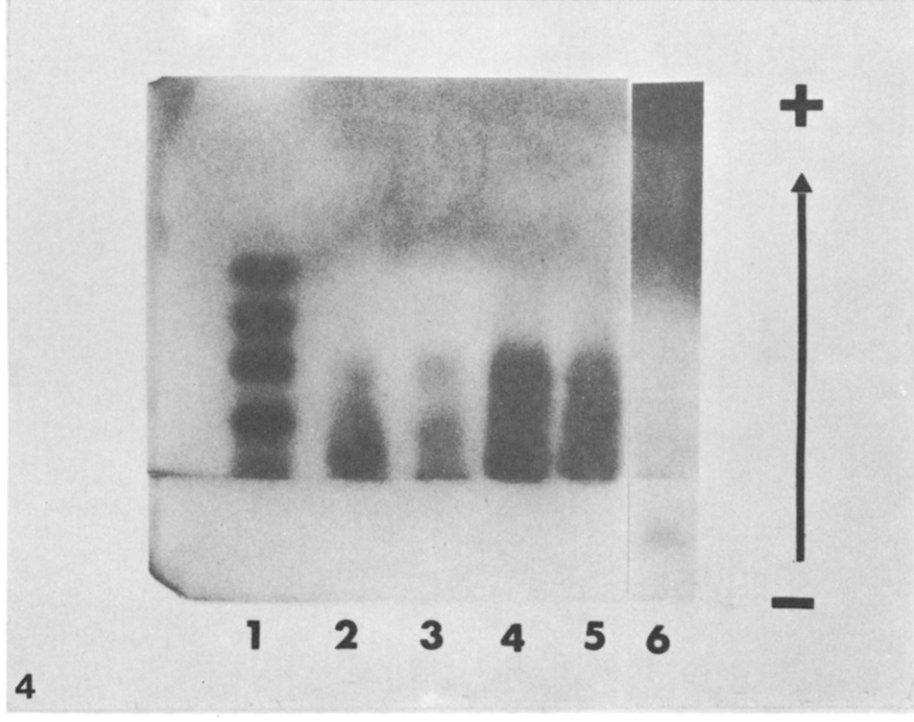


Fig. 3. *Electrofocusing of pyruvate kinase isozymes from* 1*: LF cells line.* 2*:* F_I, 3*:* F_{II}, 4*:* F_{III} *cell lines*, 5*: rat muscle*, 6*: rat liver.*

Fig. 4. *Electrophoretic pattern of aldolase in* 1*: rat brain*, 2*: LF*, 3*:* F_I, 4*:* F_{II}, 5*:* F_{III} *cells lines*, 6*: rat liver.*

(when the neoplastic transformation appeared) even with high extract concentrations. Moreover, the inhibitory effect was never found with extracts of LF hepatoma cells *in vitro*, or *in vivo*, even at very high concentrations. We have previously shown that arginase which is known to be present in normal liver but absent in hepatoma, is not the inhibitory factor of liver extracts [15].

DISCUSSION

Most studies of transformation *in vitro* concern fibroblastic cell lines. Some of the morphologic criteria often used to score for transformation of these cells in culture, cannot be applied to epithelial cultures.

Few studies have reported spontaneous transformation of rat liver epithelial cells.

In 1965, Katsuta *et al.* [27] obtained neoplastic transformation by stress in the "nagisa" zone; later Sato *et al.* (1968) [28], Oshizo *et al.* (1972) [29], Allen and Yipe *et al.* (1976) [30] also described spontaneous transformations of these cells, Borek (1972) [31] obtained transformation by nutritional stress alone.

Weinstein *et al.* (1975) [32], Karasaki *et al.* (1976) [7] reported viral particles in their neoplastic "spontaneous" transformed cells.

In this present study, we can follow the spontaneous transformation of different independently isolated epithelial cell strains derived from normal rat liver, and we define the frequency and the sequential appearance of modifications which generally occur during neoplastic or non-neoplastic transformation.

"Normal" epithelial liver cells in culture were flat, strongly adhering to their substrate and their neighboring cells; they grew as monolayer without overlapping. When they spontaneously transformed, we always observed as in neoplastic cells an early decrease of their adhesiveness to the substrate as noted by Sanford *et al.* [33], a progressive overlapping and an increase in microvilli as Allen *et al.* [30]. Our results concerning degree of saturation density agree with those of Borek [31] and Weinstein *et al.* [32]. However, differences between one strain and another appeared: if the F_{III} cells, (transformed but not yet neoplastic) show no piling-up, we can observe as for neoplastic F_I cells, a decrease of adhesiveness and a net increase of saturation density, but at a later passage than for F_I.

Thus, in agreement with Sandorf *et al.* [7], it seems that a high growth rate does not necessarily indicate a neoplastic conversion in the cells.

The changes of the specific activities of the studied enzymes which appeared, especially in F_I and LF cell strain, could not represent an early event of transformation since no significant difference appeared between the "normal" F_{II} strain and the "transforming" F_{III} strain. In the same way, Karasaki *et al.* [35] recently reported no ATPase activity at the time the cell strains studied became tumorigenic.

Ability to grow in soft-agar has been reported by many authors, providing a simple and objective method of scoring for neoplastic transformed epithelial cells. It is well known that only the tumorigenic cells grow in soft agar. We have verified that the F_I strain and the LF hepatoma strain which could induce tumors by injection into newborn rats could grow in soft agar. However, this last test could not represent an early event of transformation since no colonies were produced in agar by the F_{III} strain. In addition, we have observed that with Williams' medium, instead of MEM medium, the LF neoplastic hepatoma cell strain fails to grow in agar. It shows the importance of components of medium or of serum in the growth of the culture in soft agar. Eagle *et al.* [36] also reported that a neoplastic transformed kidney-cell strain, which normally produced tumors, did not grow in soft agar. Schaeffer and Polifka [37], also noted that, while cells produced tumors, there was no growth in agar. Thus, it seems that a negative test of growth in soft agar does not provide evidence of a non-transformed state of the F_{III} cells strain.

"Spontaneously" transformed epithelial F_I and F_{III} cell strains remained different from the LF hepatoma cell strain, which showed the highest saturation density and piling-up. The increase of specific activities of different enzymes, especially LDH, appeared higher in LF hepatoma cells than in F_I or F_{III} cells. In addition, when these LF cells were injected into rats, tumors were always produced in a short time with the characteristics of differentiated hepatoma. For the F_I cell strain, the tumorigenic properties were present but tumors appeared much later and were not so differentiated. They were not yet present for F_{III}.

It could be supposed that the differences observed between the different liver-cell strains studied, and also between the spontaneous liver-transformed epithelial cells and

hepatoma cells in culture, represented different steps in carcinogenesis. Ability to grow in agar and tumorigenic properties could be the late and progressively acquired characteristics of transformation.

The parenchymal origin of these epithelial cells has to be discussed. Epithelial cells prepared from normal rat, could have been derived from either the liver parenchyma or from bile duct epithelium. Since it is difficult to distinguish them in culture, the demonstration of liver specific, biochemical functions in these cultured cells could be theoretically the best marker for the origin of these cells.

Specific aldolase B and pyruvate kinase L isozymes were absent in all our cell strains studied before and after transformation. But, there are no published evidence, except Chessebeuf's data [38], that non-tumorigenic epithelial cell strains initiated from normal rat liver conserve similar specific adult liver functions. Consequently in our systems, these enzymes cannot be used either as a "marker" of epithelial cells or as a test of neoplastic transformation.

Some authors have reported a protein "inhibitory factor" as specific to normal liver tissue. Evidence for its presence has been shown in the F_I cell strain. It disappears thereafter with occurrence of transformation. However, further studies are required to determine whether or not the transformation and/or cell growth play a role in the disappearance of this hepatic specific "inhibitory factor".

The inducibility of TAT by corticoids has been reported by a number of investigators, as a parenchymal specific property. This property is conserved in many hepatoma cell strains. Weinstein *et al.* [32] did not find it in normal epithelial cells strains. Assays with F_I, F_{II}, and F_{III} strains indicated that the F_{II} and F_{III} strains lacked dexamethasone-inducible tyrosine aminotransferase but that this enzyme could be induced in neoplastic transformed F_I cells.

It seems that under the culture conditions the lack of homeostatic regulators such as hormonal and immunologic controls, the state of continued growth and aging can undergo different modifications and loss of functions. We have reported that addition of hydrocortisone hemisuccinate to the medium is required for the maintenance of hepatocytes in culture [3]. This observation was recently confirmed by Laishes and Williams [39] who suggest that complex media may have to be devised to maintain functioning epithelial cells in culture.

What does "spontaneous" neoplastic transformation mean? It seems, for Weinstein *et al.* [32], that viral particles found in a hepatoma cell and in a spontaneous transformed cell strain, might play a causative or cofactor role in the induction of hepatomas. However, in our systems, using ultrastructural studies, no evidence for oncogenic virus or PPLO particles was seen; they could not be involved in the spontaneous transformation. In addition, a difference in the susceptibility in rat strains could not be involved either, since the transformed F_I and F_{III} cell strains came from two different strains (Fischer and Commentry) and the "normal" F_{II} cell strain from the Fischer strain, as did F_I. There is perhaps, an effect due to the calf serum used in our cultures. It is well known that some components of calf and horse serum, as compared with fetal calf serum, accelerate neoplastic transformation [35].

But it appears that we can still say now, like Hayflick [40], that "spontaneous" only means that we are ignorant of the cause.

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Growth of Canine Lymphosarcoma in X-Irradiated and Non-Irradiated Athymic (Nude) Mice*

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Abstract—*Lymphosarcoma cells from 8 dogs failed to grow in Nude mice. Following whole-body X-irradiation of Nude mice cells from another canine lymphosarcoma grew successfully and were subsequently transplanted to both irradiated and non-irradiated Nude mice. When injected subcutaneously large infiltrating tumours grew and metastasised widely.*

INTRODUCTION

MANY solid human tumours have been transplanted into Nude mice (Nu) as has Burkitt's lymphoma [1] but Nu mice rarely developed tumours after i.m., i.p. or s.c. inoculations of other human lymphomas. Intracranial hetero-transplantation of various non-Burkitt lymphomas however yielded 90% "takes" [2].

Canine osteosarcoma, mammary carcinoma and melanoma have been successfully transplanted [3]. A canine mast cell leukaemia was transmitted as an ascites tumour [4].

In mice it has been shown that a majority of BALB/c lymphoid tumours grow at a significantly reduced rate in BALB/c Nu mice when compared with growth in BALB/c mice [5]. Irwin *et al.* [6] were not able to transplant bovine lymphosarcoma in Nu mice unless whole body irradiation had been previously given.

In the present experiments canine lymphosarcoma failed to grow in Nu mice but was successfully transplanted into X-irradiated Nu mice. Further passages grew both in irradiated and non-irradiated animals.

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MATERIALS AND METHODS

Lymphosarcoma

Eleven dogs histologically diagnosed as multicentre lymphosarcoma, one of which was also leukaemic, had one entire lymph node, usually either the prescapular or popliteal excised under anaethesia. Tumour tissue was chopped into 1–2 mm fragments using crossed scalpels and was then sieved through a 50 μm stainless steel mesh using Hanks' balanced salt solution. Viability of cells harvested in this way is usually >70% as assessed in trypan blue exclusion tests.

Nu mice

The mice were obtained from the Medical Research Council, Carshalton 5 yr ago and have been kept as a closed colony under barrier-maintained conditions [7]. Mice used in the experimemts were between 8 and 12 weeks of age. Whole body X-irradiation when given was from a Marconi Radiotherapy machine (250 K/V 15 Ma, Thoreus filter 0.44 mm SN +0.25 mm CU +1 mm Al). Lymphosarcoma cells were injected 4–24 hr after irradiation.

Transplantation

Cells from each tumour were injected into one to four mice dependent on availability of animals. In first transplant experiments a total of 28 mice were injected. The usual route of injection was s/c over the back but 9 mice were injected i/p.

The number of cells injected was usually 10^7–10^8.

In the lymphosarcoma successfully transplanted into X-irradiated Nu mice further transplants were made using a similar technique to that used with the primary tumour. Four passages were made using a total of 12 irradiated and 12 non-irradiated mice.

Tissue culture

Aliquots of freshly prepared cell suspensions were placed in RPMI 1640 medium supplemented with 20% foetal calf serum and incubated at 38°C.

RESULTS

In 20 non-irradiated mice injected with lymphosarcoma cells from 8 dogs no tumour growth was detected. Most mice lived between 90 and 330 days following the injection but 6 mice lived 37–62 days only.

Two mice which had received 700R whole body X-irradiation and lymphosarcoma cells died 4 days later. Two other similarly irradiated mice injected with a different lymphosarcoma died after 7 days. Two mice receiving 300 rad and 2 mice receiving 500 rad were later injected s/c with lymphosarcoma cells from a 5 yr old female Alsation. Tumour growth was apparent 3 weeks later and developed into a large subcutaneous mass.

In further passages both into X-irradiated and non-irradiated mice growth occurred in both irradiated and non-irradiated mice after the second passage. Because of the diffuse nature of the tumours spreading subcutaneously measurements of growth rate were not possible but no obvious differences in the growth in irradiated and non-irradiated animals occurred. Tumours reached a weight of 5 g without ulceration within 6 weeks by the fourth passage and regression was not seen in any of the mice injected.

In the third and later s/c passages tumour was also found to have invaded the peritoneum and metastases were found in the axillary lymph nodes, lungs, kidney and heart.

Histologically the lymph node from the original dog showed complete loss of normal architecture and was replaced by a sheet of closely packed lymphoid cells with some lymphoblastic types. Mitotic figures were common (Fig. 1). The transplanted tumour showed similar features both in the subcutaneous mass and at distant sites (Fig. 2). Cells in primary cultures and cultures from transplanted tumour remained viable *in vitro* for a few days only and long-term cultures were not established.

DISCUSSION

It is generally recognised that there is a resistance to the growth of lymphomas in Nu mice when compared with other tumours. Various hypotheses as to why this occurs have considered the possible effects of tumour inhibition by macrophages, lack of some growth factor, residual T cell activity [8–10] or the role of natural tumour antibodies [11].

Campanile *et al.* [12] have reported data indicating a relatively strong radioresistant transplantation resistance against allogeneic tumour cells in Nu mice. They were not able to determine to what extent this resistance is related to the Hh system or to natural cytotoxicity or to other immunological factors.

Warner *et al.* [5] in their studies on inhibition of growth of lymphoid tumours in syngeneic Nu mice have put forward an explanation that these mice have increased levels of non T cell immunity to certain virus associated lymphoid tumours. Their results correlate the *in vitro* lytic activity of haematopoietic cells from Nu mice with the *in vivo* growth pattern of various virus and non-virus associated tumours.

The article by Warner *et al.* [5] refers specifically to murine tumours. Protection against transplantation of xenogeneic lymphoid tumours may be due to natural killer cells directed at interspecific glycoprotein (gp70) determinants or other interspecific oncornaviral antigen determinants expressed on these cells. Wecker *et al.* [13] have shown that gp70 can be expressed in the absence of complete virus expression and can also be found on normal murine lymphoid cells that have been activated immunologically. Burkitt's lymphoma suspect for Herpes viral aetiology is easily transplantable and Marek's disease of proven Herpes viral aetiology has been transplanted into Nu mice [14]. This is in contrast to bovine lymphosarcoma of known oncornaviral aetiology. The belief that natural killer cells may be directed in the manner described gives further support for an oncornaviral aetiology of canine lymphosarcoma.

In our cases of transplanted lymphosarcoma there were widespread tumour deposits following subcutaneous inoculation and it appears

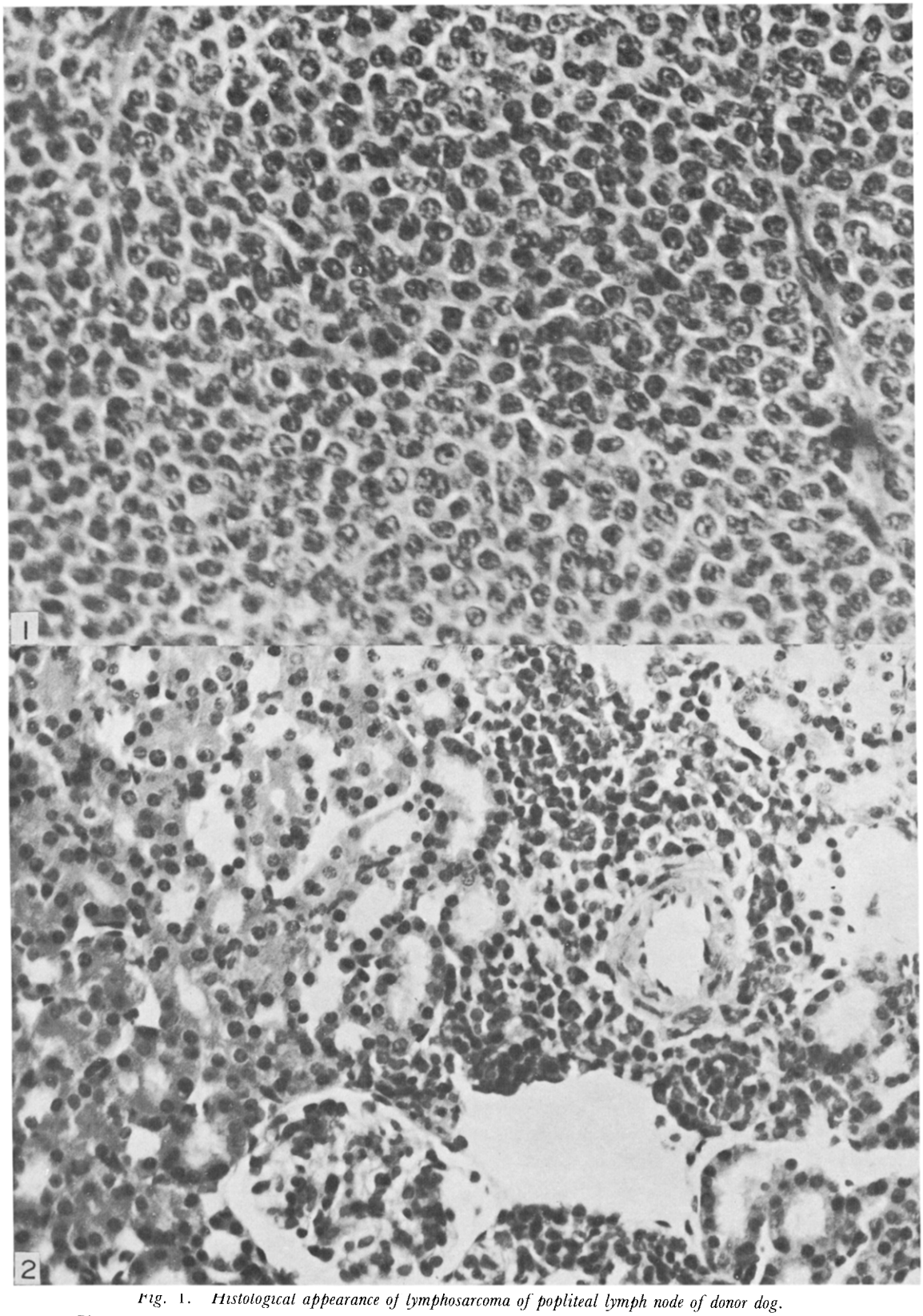

Fig. 1. Histological appearance of lymphosarcoma of popliteal lymph node of donor dog.

Fig. 2. Lymphosarcoma in kidney of Nu mouse injected s/c with lymphosarcoma cells—fourth passage. Tumour was also found in the lungs and heart.

likely that this was true metastatic spread. Metastasis is rare in tumours transplanted into Nu mice. In the lymphomas described by Epstein *et al.* [2] which were injected intracranially 2 of 21 showed extra cranial spread. Nodular lesions developed 7–8 months after implantation but they resembled diffuse mixed histiocytic and lymphocytic leukaemia rather than the original Hodgkins cases. They contained foci of cells which reacted positive with anti-human immunofluorescent reagents.

Price *et al.* [15] and others have reported the recovery of murine xenotropic type C viruses from cell cultures of hetero-transplanted human tumours in Nu mice. Epstein *et al.* [2] have considered the possibility that these viruses may enhance the growth potential of human lymphoid cells in Nu mice. Only limited E.M. studies have been made on our transplanted canine lymphosarcoma tumours and no viral particles seen but C type particles have been found in transplanted canine osteosarcoma tumours.

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Preliminary Evaluation of a Phase I Clinical Study of Quelamycin

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Abstract—*We have performed a preliminary evaluation of a phase* I *clinical trial of quelamycin (triferric adriamycin) with* 20 *patients. Quelamycin has appeared to have a good general tolerance, the main toxicity being characterized by leucopenia and alopecia. The maximum dose was* 225 mg *equivalent*/m^2 *and the drug produced* 4 *objective responses out of* 16 *evaluable patients.*

INTRODUCTION

QUELAMYCIN is triferric doxorubicin, a metallic derivative of adriamycin made by chelation of adriamycin with three ferric ions at neutral pH [1, 2]. A preclinical evaluation of quelamycin indicates that this derivative has less general, hematological and cardiac toxicity than adriamycin while it maintains similar antitumor action [1–4]. This report presents the preliminary evaluation of a phase I clinical trial with quelamycin carried out in Madrid by the Oncological Group of Work formed by the clinical oncologists of the six major Madrid hospitals.

MATERIALS AND METHODS

Quelamycin was synthesized by Dr. M. Gosálvez and was liophylized and sterilized by Montedison Farmaceutica, S.A. (Barcelona) in vials of 25 mg equivalent of activity to be reconstituted with 10 ml of water for intravenous injection. The vials were used within the first 6 months after the manufacturing date and the injection of the drug was performed slowly and directly into the vein. One milligram equivalent of quelamycin is the quantity of triferric adriamycin (1.25 mg) which has the same activity in inhibiting DNA synthesis as 1 mg of adriamycin. Each milligram equivalent of quelamycin, upon the dissociation of the chelate by heating with EDTA, yields 1 mg of adriamycin [4].

Patients

Patients with histologically confirmed advanced cancer, not amenable to conventional treatment, were eligible for inclusion in the study. All patients were recognized to have a life expectancy of $\geq$12 weeks and a Karnofsky performance status of $\geq$30%. Patients were admitted to the study if they had no other chemotherapy for 8 weeks previously or radiotherapy for 12 weeks previously and if they had not previously received adriamycin. All patients had received prior chemotherapy. Other requirements included adequate cardiac (normal EKG) and renal function (BUN 25 mg/100 ml), serum bilirubin of less than 1.5 mg/100 ml) serum glutamic oxaloacetic transaminase (SGOT) of less than 100 i.u., pretreatment white blood cells wbc and platelet counts in excess of 5000 and 100,000 cells/mm^3, respectively. Physical examination, determination of wbc, differential, platelet, reticulocyte counts and electrocardiograms were performed weekly. Every three weeks SGOT, BUN, creatinine, bilirubin, serum iron and X-rays were performed. Bone marrow aspiration was performed initially and when indicated. Tumor measurements using the product of the greatest horizontal and the greatest vertical diameter were obtained weekly or biweekly. In assessing tumor responses, objective response was considered when the patient had a dimunition of $\geq$50% of tumoral masses. Subjective

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response was considered when the patient had a dimunition of more than 25% but less than 50% of tumoral masses. Stable disease was considered when the tumor progression arrested.

The starting dose based on preclinical toxicology studies [4] was in all cases 75 mg equivalent/m^2 of body surface area given as a direct, slow, intravenous injection. Doses were repeated every three weeks and were escalated according to individual hematological tolerance which was very variable. A minimum wbc of 3000/mm^3 was required before starting the next dose. If with the first dose the nadir of neutrophils was not less than 1500 cells/mm^3, the second dose was increased to 125 or 150 mg equivalent/m^2. If doses of 150 mg/m^2 were tolerated with a nadir of more than 1500 leucocytes/mm^3, the following dose was escalated to 200 or 225 mg equivalent/m^2.

RESULTS

Of the 20 patients who entered into the study, all were evaluable for toxicity and 16 for antitumor effect; the other 4 received only one cycle of therapy and later either died or rejected treatment, or the evaluation was insufficient. Immediately following the dose of quelamycin, all patients had vomiting and nausea, 4 patients mentioned a metallic after-taste and 7 patients had fever (37–39°C) and chills. In 4 patients the urine was red consequently to the drug. All patients had variable neutropenia which was maximal on the 14th day after the injection of quelamycin and was dose related (Table 1) and not cumulative. Only one case showed thrombocytopenia (75,000 platelets/mm^3). Alopecia was observed in 12 cases and was complete in all after the second dose. No other toxicity was observed. No alteration of any biochemical parameter including enzymes and iron in serum was observed. In spite of the fact that three patients surpassed cumulative doses of quelamycin superior to 500 mg equivalent/m^2 (600, 750 and 1700 mg equivalent/m^2 respectively), no electrocardiographic (EKG done weekly) or clinical signs of cardiotoxicity were observed in any of the patients at any of the doses. The patient that reached 1700 mg equivalent/m^2 died of tumoral progression and at necropsy no histological signs of cardiomiopathy were observed in the heart.

The signs of toxicity were always reversible and no case of death due to the medication was encountered. Although the primary purpose of the study was to study the toxicity, antitumoral objective responses (Table 2) were observed in 4 cases. The antitumoral action of quelamycin was evident from the very first dose of 75 mg equivalent/m^2 and did not apparently increase with the augmentation of the dose. The longest period of treatment reached was 7 months in an epidermoid lung carcinoma that maintained a subjective response all throughout.

DISCUSSION

It is evident that quelamycin can be administered as a single dose every three weeks at doses of 150 mg/m^2 without causing severe bone marrow suppression. However, there is a factor of variable individual tolerance to the drug and there were cases in which doses of 75 mg/m^2 caused bone marrow suppression and there was a case in which, with doses of 225 mg/m^2, the bone marrow effect was moderate. In spite of the very advanced state of all neoplastic

Table 1. *Hematological toxicity*

			Nadir of neutrofiles		
Dose	No. cycles	No. patients	Superior value	Inferior value	Mean ± S.D.
75 mg/m^2	24	20	7330	582	3029 ± 1646
125 mg/m^2	5	3	2890	1200	1798 ± 699
150 mg/m^2	9	9	2021	528	1335 ± 528
225 mg/m^2	5	2	1985	50	982 ± 409

Table 2. Tumoral responses

Diagnosis		Number of cases	Objective response	Subjective response	Stable disease	Progressive disease
Lung carcinoma	epidermoid	5	1	1		3
	anaplastic	2	1			1
	adenocarcinoma	1				1
Gastric carcinoma		5	1	1		3
Colon carcinoma		1	1			
Maxilar epidermoid carcinoma		1				1
Resistant breast cancer		1			1	
		16	4	2	1	9

patients subjected to treatment, a good general tolerance to quelamycin was evident either immediately or delayed.

Quelamycin showed tumoral response in 4 cases. These effects are an indication that quelamycin has antitumoral action, but we can not conclude from our limited data whether its antitumor effect is equal or superior to the antitumor effect of adriamycin. Similarly, there is not yet sufficient data to ascertain a lower cardiotoxicity with quelamycin. However, if the relation of antitumor potency of quelamycin with adriamycin were equivalent mg–equivalent to mg, quelamycin could represent an advantage because it is three times less acute dose limited than adriamycin. It is, on the other hand, noteworthy that, among the responses, there was a colon carcinoma and an anaplastic lung carcinoma (large cells), two kinds of tumors that, in our hands, are resistant to adriamycin.

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In Vitro Effects of Methotrexate, 5-Fluorouracil, Cyclophosphamide and Vincristine Sulphate on the Metabolism of Induced Mammary Cancers in Rats*

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Abstract—*In function of concentrations* 0.001–100.0 μg *methotrexate added to the incubation medium, the* [2-^{14}C]*glycine incorporation into the proteins of experimental mammary cancer tissue of rats became progressively reduced. Only the highest concentrations of methotrexate reduced the* [1-^{14}C]α*-aminoisobutyric acid uptake by the cells.*

In the present in vitro *system, 5-fluorouracil only inhibited the* [6-^{3}H]*thymidine incorporation into DNA without affecting either glycine incorporation or the* α*-aminoisobutyric acid uptake. Cyclophosphamide impaired the glycine incorporation into the proteins as well as the thymidine incorporation into DNA in function of increasing amounts added, while the highest concentrations applied resulted in an increased* α*-aminoisobutyric acid uptake by the cells.*

With vincristine sulphate addition to the incubating tumour-slices, glycine incorporation and α*-aminosoisobutyric acid uptake were reduced which was particularly noticeable at the highest dose levels.*

INTRODUCTION

HORMONAL treatment and chemotherapy of advanced and metastatic breast cancer by a combination of different antineoplastic agents resulted in important tumour regression [1–3]. In the mean time considerable progress has been made in the understanding of the mechanism of action by which the different agents affect the normal and the tumour cells [4, 5].

Apart from the established knowledge of methotrexate and 5-fluorouracil effects on thymidylate synthetase and of vincristine and vinblastine sulphate on the depolymerisation of spindle microtubules, the alkylating action of cyclophosphamide and the generally accompanying cytotoxic effects of most of these compounds are still not sufficiently understood.

In an attempt to approach some of the problems concerning the mechanism of action, the *in vitro* effects of methotrexate, 5-fluorouracil, cyclophosphamide, and vincristine sulphate, on the metabolism of experimental mammary carcinoma of rats, have been examined.

MATERIALS AND METHODS

Adult female locally inbred Sprague–Dawley rats, weighing 180–200 g were fed on an *ad libitum* Purina Chow diet and having free access to pure drinking water were each treated over a period of 5 weeks with a weekly stomach intubation of 0.5 ml solution of 7.12-dimethylbenz(a)anthracene (DMBA) (Fluka) 1% in olive oil [6].

Tumours appeared along the mammary lines from the sixth week after the treatment was completed. Mammary tumours measuring at least 1 cm and up to 3 cm in diameter were excised, freed from connective tissue and fat and cut in slices with a Stadie–Riggs microtome. Samples of 200 mg wet weight were placed in Erlenmeyer flasks and

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incubated in 5 ml Krebs–Ringer bicarbonate buffer, pH 7.2, previously gassed with 95% O_2–5% CO_2, for 1 hr in a shaking waterbath at 37°C. The incubation medium contained radioactive labelled precursors as well as progressively increasing doses of various anti-neoplastic agents. The addition of 1 μCi [2-^{14}C]glycine (sp. act. 10 mCi/mmole) to the medium allowed to follow the incorporation into the proteins. The effects on DNA synthesis were evaluated by the addition of 10 μCi [6-^{3}H]thymidine (sp. act. 10 Ci/mmole) to the medium.

By the incubation with 2 μCi [1-^{14}C] α-aminoisobutyric acid (AIB) (sp. act. 58 mCi/mmole) (Radiochemical Centre, Amersham, England) some aspects of transport mechanism and cell membrane integrity were examined [7].

Methotrexate (Lederle), 5-fluorouracil (Roche Products), cyclophosphamide (Mead Johnson Co.), and vincristine sulphate (Eli Lilly & Co.), were applied in concentrations of 0.001, 0.01, 0.1, 1.0, 10.0 and 100.0 μg in 5 ml buffer.

After the incubation with [2-^{14}C]glycine and [6-^{3}H]thymidine, 10 ml ice-cold 20% trichloroacetic acid (TCA-British Drug Houses. AR grade) were added to the Erlenmeyer flasks to precipitate the proteins. The denatured proteins were twice washed with 5 ml TCA 5% and subsequently twice heated for 15 min in 5 ml TCA 5% at 90°C to extract the nucleic acids. After incubation with [1-^{14}C] α-aminoisobutyric acid, the reaction was interrupted by the addition of 20 ml ice-cold physiological saline solution to the medium, followed by two more washes of 10 ml saline, to remove extracellular labelling. The protein precipitates after incubation with [2-^{14}C] glycine and the dried down hot TCA extracts after [6-^{3}H] thymidine incubation as well as the tumour tissue after [1-^{14}C] α-aminoisobutyric acid incubation, were dissolved in 3 ml of hydroxyde of hyamine-10-X (Packard Instrument Co.) by heating overnight in a waterbath at 60°C.

After the addition of 15 ml scintillator liquid to the hyamine solutions, the radioactivity was counted in a Liquid Scintillation Spectrometer (720 series—Nuclear Chicago) [7]. The incorporated radioactivity was expressed as DPM per 100 mg protein.

Standard errors of the mean and Student's *t*-test were calculated. The protein contents of the different tumours were determined colorimetrically [8]. The tumours used were histologically analysed.

RESULTS

Histological analysis of the different mammary tumours used revealed a complex adenocarcinoma type structure with some secretory characteristics.

The protein content of these cancers varied

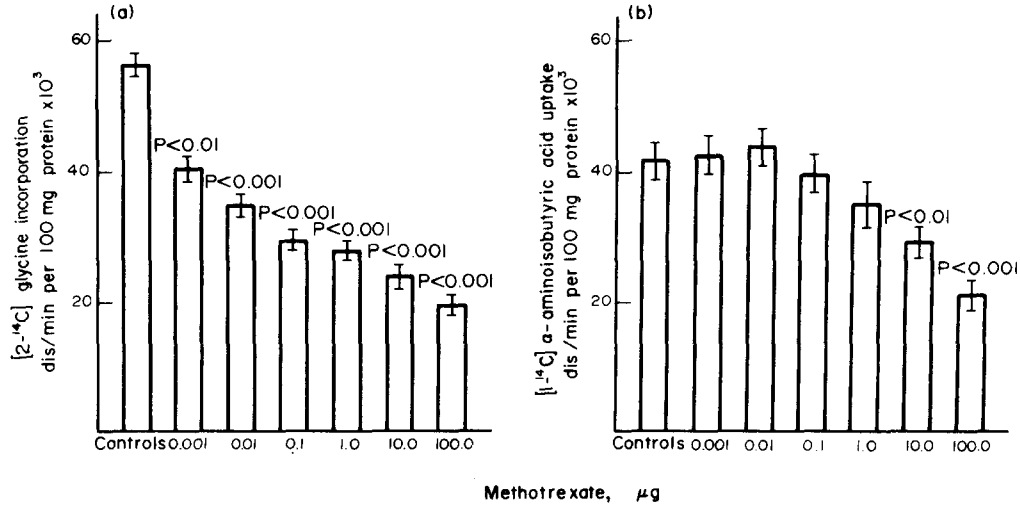

Fig. 1. The effects of in vitro *methotrexate treatment on mammary carcinoma tissue. Aliquots of* 200 mg *tumour tissue were incubated at* 37°C *for* 1 hr *in* 5 ml *Krebs–Ringer bicarbonate buffer containing different concentrations of methotrexate, as well as the radioactive tracers (Materials and Methods). The incorporation of* [2-^{14}C] *glycine into the proteins (a) and the uptake by the cells of* [1-^{14}C] α-*aminoisobutyric acid (b) are expressed as dis/min per* 100 mg *protein for each dose level of methotrexate used. The values represent the mean of* 8 *experiments* ±S.E.M. *P values between the controls and the treated samples are given when significant.*

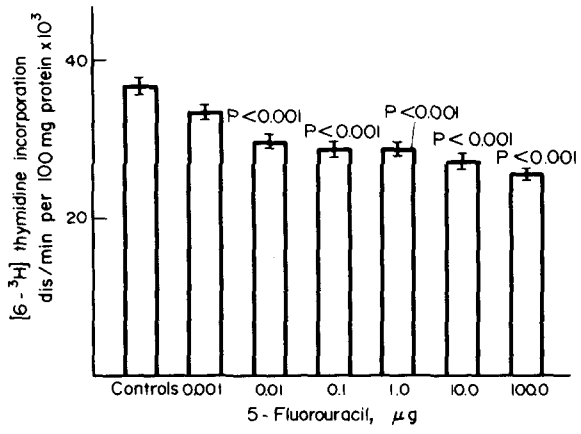

Fig. 2. The effects of in vitro *5-fluorouracil treatment on mammary carcinoma tissue. Aliquots of* 200 mg *tumour tissue were incubated at* 37°C *for* 1 hr *in* 5 ml *Krebs–Ringer bicarbonate buffer containing different concentrations of 5-fluorouracil, as well as the radioactive tracers (Materials and Methods). Each value representing the* [6-^{3}H] *thymidine incorporation into DNA expressed as dis/min/*100 mg *protein in function of the 5-fluorouracil dose used, is the mean of* 8 *experiments* ±S.E.M. P *values between the controls and the treated samples are given when significant.*

between 85 and 110 mg/g of wet weight.

Methotrexate treatment inhibited the [2-^{14}C] glycine incorporation into the proteins in function of the dose level applied. The smallest methotrexate dose used 0.001 μg reduced the incorporation by 28.0% ($P<0.01$) while 100 μg methotrexate resulted in a glycine incorporation of only 35.0% ($P<0.001$) of the non-treated control samples (Fig. 1a). The [6-^{3}H] thymidine incorporation into DNA amounting to 25,800±749 dpm/100 mg protein for the control samples was not affected by methotrexate treatment. Only the highest concentrations of 10.0 μg and 100.0 μg methotrexate reduced the [1-^{14}C] α-aminoisobutyric acid uptake by the cells respectively by 29.0% ($P<0.01$) and by 48.6% ($P<0.001$) (Fig. 1b).

With 5-fluorouracil treatment only the [6-^{3}H] thymidine incorporation into DNA became depressed, reaching 80.8% ($P<0.01$) of the control values with 0.01 μg and 70.1% ($P<0.001$) with 100.0 μg while the [2-^{14}C] glycine incorporation and the [1-^{14}C] α-aminoisobutyric acid uptake were not significantly affected (Fig. 2). Cyclophosphamide, 0.01 μg decreased the [2-^{14}C] glycine incorporation into the proteins by 28.2% ($P<0.01$) reaching a maximal inhibition with 10.0 and 100.0 μg when only 59.4% ($P<0.001$) of the control values were incorporated (Fig. 3a). Even with a low dose of 0.001 μg, a significant 15.7% ($P<0.01$) inhibition of the [6-^{3}H] thymidine incorporation into DNA was observed, reaching 22.9% ($P<0.001$) with the highest dose levels (Fig. 3b). A dose level of 10.0 μg cyclophosphamide increased the [1-^{14}C] α-aminoisobutyric acid uptake into the cells by 53.5%($P<0.01$) while with 100.0 μg the uptake was even more than doubled to 211.0% ($P<0.001$) of the controls (Fig. 3c). Vincristine sulphate treatment affected

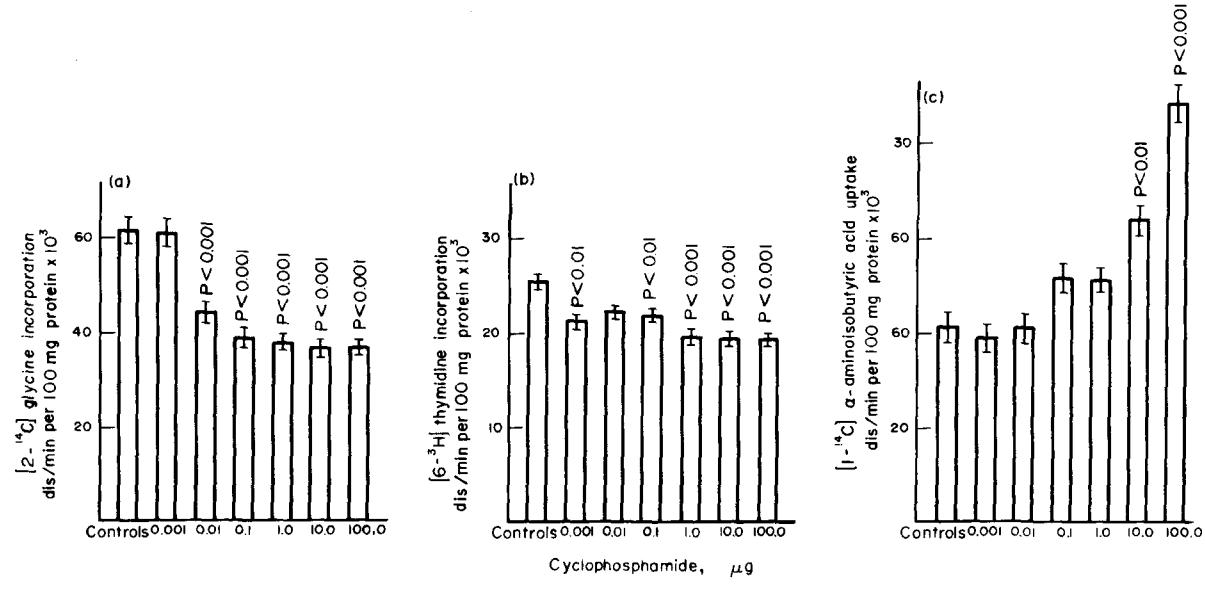

Fig. 3. The effects of in vitro *cyclophosphamide treatment on mammary carcinoma tissue. Aliquots of* 200 mg *tumour tissue were incubated as described in Materials and Methods. The incorporation of* [2-^{14}C] *glycine into the proteins (a), of* [6-^{3}H] *thymidine (b) into DNA and the* [1-^{14}C] α-*aminoisobutyric acid (c) uptake by the cells are expressed in function of the cyclophosphamide treatment. Each value represents the mean of* 8 *experiments* ±S.E.M. P *values between the controls and the treated samples are given when significant.*

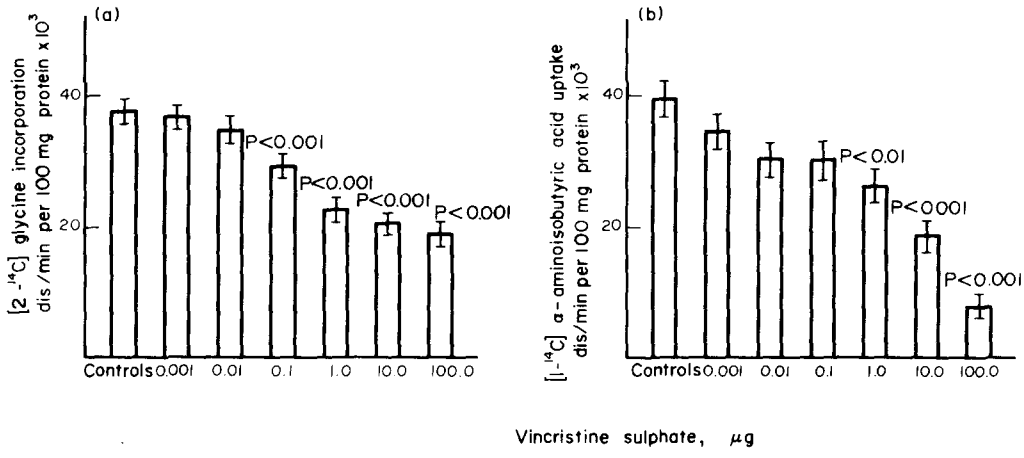

Fig. 4. The effects of in vitro *vincristine sulphate treatment on mammary carcinoma tissue. Aliquots of* 200 mg *tumour tissue were incubated as described before. The incorporation of* [2-^{14}C] *glycine into the proteins (a) and the* [1-^{14}C]α-*aminoisobutyric acid (b) uptake by the cells are expressed in function of the vincristine sulphate dose level applied. Each value represents the mean of* 8 *experiments* ±S.E.M. P *values between the controls and the treated samples are given when significant.*

the [2-^{14}C] glycine incorporation with 0.1; 1.0; 10.0 and 100.0 μg by a reduction of 21.7% ($P<0.001$), 40.0% ($P<0.001$), 45.7% ($P<0.001$) and 48.2% ($P<0.001$) without effect on the [6-^{3}H] thymidine incorporation into DNA (Fig. 4a). The [1-^{14}C] α-aminoisobutyric acid uptake was inhibited with 1.0 μg by 33.7% ($P<0.01$) with 10.0 μg by 53.0% ($P<0.001$) and with 100.0 μg by 79.8% ($P<0.001$) (Fig. 4b).

DISCUSSION

The absolute values of precursor incorporation sometimes differ considerably from one batch of tumour tissue to another which depends on the structure and the differentiation of the tissue leading to varying amounts of protein content. As the effects on incorporation increase almost linearly for up to 6 hr in function of incubation time, it is assumed that drug as well as radioactive precursors penetrate the tumour cells homogeneously.

Methotrexate inhibits thymidylate synthetase probably by interfering with the enzyme stabilisation as well as dihydrofolate reductase. As tetrahydrofolate becomes depleted a reduction of folate dependent purine and protein synthesis occurs [9–11]. Thymidine and deoxyadenosine are known to reverse the toxicity of methotrexate which does not affect the *in vitro* incorporation of [6-^{3}H] thymidine into the DNA chain [12]. The [1-^{14}C] α-aminoisobutyric acid uptake by the cells being reduced by the highest methotrexate levels used seems due to a toxic effect on membrane transport mechanism, which accentuates the inhibition of [2-^{14}C] glycine incorporation into the proteins already affected by the tetrahydrofolate depletion which interferes with glycine metabolism [13–15].

The inhibitory effect of 5-fluorouracil on thymidylate synthetase should be reduced or even neutralized by the addition of thymidine to the incubation medium [12, 16]. However the *in vitro* [6-^{3}H] thymidine incorporation into DNA becomes progressively reduced in function of the added 5-fluorouracil dose which may be explained by a competitive incorporation of this base analogue into DNA eventually through mutagenesis leading to pathological gene transcription. This effect confirmed by the incubation of the tissue with 5-fluoro [6-^{3}H] uracil, is also occurring in RNA where it is only considered to be of secondary importance [17].

The glycine incorporation into the proteins continues unchanged in spite of increased 5-fluorouracil treatment which implies the pre-existing messenger RNA to be responsible for the immediate protein synthesis in the presence of an intact membrane system. The alkylating cyclophosphamide is now considered to be converted in the liver by a microsomal oxydase system to 4-hydroxycyclophosphamide and its tautomeric aldophosphamide which through further still uncertain transformations may lead to several not yet recognised active forms [18, 19]. The cytotoxic effect of cyclophosphamide however may be produced by conversions in the tum-

ourcell itself[5]. This cytotoxic activity, although unexplained and not further explored in this study, seems responsible for the inhibition on the thymidine incorporation into DNA and on glycine incorporation into the proteins, while the increased α-aminoisobutyric acid content in the cells is most probably due to a destructive effect on the membrane structure leading to an easier passage of amino acids into the cells [20]. The metaphase arresting compounds, vincristine sulphate and vinblastine sulphate are rapidly taken up by the cells where they attach to the microtubule proteins leading to destruction of the mitotic s spindle [21–24]. In the cell they produce cytoplasmic vesicles which line up along the membranes [25]. In these short term *in vitro* experiments the observed effects of vincristine sulphate cannot be explained by the inhibitory activity on mitosis. Thymidine incorporation into DNA is not even affected. The incorporation of glycine into proteins seems related to the precipitation of certain cellular proteins as well as to an inhibition of the cellular membrane transport system which results in a decreased amino acid uptake into the cells [26].

Apart from the expected effects of the examined antineoplastic agents on protein and DNA synthesis, an important effect on the membrane integrity and transport function becomes apparent when mammary cancer cells are incubated with the highest dose levels of methotrexate, cyclophosphamide and vincristine sulphate. This membrane effect greatly contributes to the inhibition of protein synthesizing activity observed in these *in vitro* systems.

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Plasma Oestradiol and Progesterone Levels in Women with Varying Degrees of Risk of Breast Cancer

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Abstract—*Increased risk of breast cancer in pre-menopausal women correlates with sub-normal plasma progesterone values in the luteal phase of the menstrual cycle and a sub-normal excretion of urinary androgen metabolites. Plasma oestradiol values do not vary with risk. In post-menopausal women, plasma oestradiol and progesterone levels are not related to risk.*

INTRODUCTION

THERE are a number of well-known factors related to risk of breast cancer, such as age at menarche, age at first child, family history and previous breast disease. A sub-normal excretion of urinary androgen metabolites has also been found to be correlated with an increased risk of the disease [1, 2].

The relation of each of the factors to the subsequent development of breast cancer has been examined in a prospective study carried out in Guernsey over the last 16 yr. Each factor, taken singly, was significantly correlated with risk [3]. Recently, a mathematical model has been constructed in which a logistic model for the probability of developing breast cancer has been combined with a Wiebull distribution for the time of diagnosis, so that the joint influence of all the above variables on subsequent risk could be estimated [4]. Five risk groups were delineated; the lowest estimated probability of developing breast cancer was 0.010: the highest was 0.224. These estimates were calculated for women in Guernsey for whom a determination of the amount of urinary aetiocholanolone was available (1498 of the 5000 women who took part in the prospective trial). It so happened that 765 of the 1498 women had provided a specimen of blood in a second prospective trial and it was possible, therefore, to assign a risk category to each of these blood donors. A variety of hormone assays were then carried out on the plasma samples to see whether endocrine function was related to the various degrees of risk. This paper deals with plasma oestradiol and progesterone in pre- and post-menopausal women.

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MATERIALS AND METHODS

1. *Subjects*

Urine specimens were obtained from 5000 normal women in Guernsey between 1961 and 1965. Between 1966 and 1976, a further 5000 volunteers gave a 50 ml sample of blood between 13.00 and 22.00 hr. This was taken into a heparinized vacutainer: the plasma was separated by centrifugation and stored at −20°C until required for analysis. All volunteers filled in a detailed questionnaire concerning reproductive history, previous and current diseases and drug history.

The five risk categories for the 1485 women in the 1961–1965 Guernsey study are shown in Table 1, with the estimated probabilities of developing breast cancer. The number of women in each class from whom blood was obtained is also given.

Pre-menopausal women were considered to be in the luteal phase of their menstrual cycle if their blood samples were obtained after at least six-tenths (rounded up to

the nearest whole number) of their cycle had elapsed. In the case of a woman with a 28 day cycle, this would mean a blood collection on or after the 17th day: in a woman with a 24 day cycle, a blood sample on day 15 was accepted. Only women who stated that they had regular cycles were accepted and only cycles between 24–36 days in length were taken.

Approximately 80% of the subjects had cycles in the range of 28 days ± 2 days. As a second check that no bias had been introduced by the above definition of the luteal phase, the mean time between obtaining the blood and the date of the subsequent cycle was calculated. These means were 4.4, 6.0, 6.7 and 6.8 days for risk groups 0–3 respectively.

Women were defined as being in the follicular phase of their cycle if the blood was obtained between days 1 and 14. However, in 14 of these women, values of plasma progesterone in excess of 3 ng/ml were found between days 9 and 14. These women were re-classified as being in the luteal phase of their cycles, on the assumption that they had had a short follicular phase. The age and menstrual cycle characteristics of the women studied are shown in Table 2.

Post-menopausal women were defined as those who had not menstruated for at least 6 months and aged over 50 yr. Menopausal women were not included. Blood samples were not analysed if any of the volunteers were on drugs known to affect endocrine function, (e.g. contraceptive steroids, hormone replacement therapy, thyroid hormones), if menstrual cycles fell outside the limits already defined, or if information on cycles or menopause was missing. Such bloods numbered 379 of the 765 samples available.

Table 1. Risk characteristics

Risk group	Family history	Log aetio	Age menarche	Age 1st birth	Estimated probability	No. of blood samples available: Age 30–49	No. of blood samples available: Age ≧50
0	0	0	0	0	0.010	24	7
	0	0	1	0	0.019		
	0	1	0	0	0.022	85	43
1	1	0	0	0	0.021		
	0	0	0	1	0.028		
	0	1	1	0	0.043		
	1	0	1	0	0.042		
	1	1	0	0	0.047	93	53
2	0	0	1	1	0.054		
	0	1	0	1	0.060		
	1	0	0	1	0.059		
	1	1	1	0	0.090		
	0	1	1	1	0.116	35	44
3	1	0	1	1	0.113		
	1	1	0	1	0.125		
4	1	1	1	1	0.224	1	1

Risk groups 0–4 are women with 0–4 risk factors positive. A zero denotes a favorable state: 1 denotes a positive risk factor. The two women in risk group 4 have been included in risk group 3 in all subsequent tables. Log aetio refers to the lorarithms of the amount of aetiocholanolone excreted, expressed in μg/24 hr.

Table 2. Age and menstrual cycle characteristics of women in the four risk groups

Risk group	N	Mean age	Mean cycle length	Mean day follicular phase	Mean day luteal phase
0	24	39.8 ± 4.6	27.1 ± 3.0	8.6 ± 4.2	21.9 ± 4.5
1	85	39.2 ± 3.7	27.0 ± 2.0	6.8 ± 3.5	19.6 ± 4.4
2	93	40.1 ± 4.5	27.2 ± 1.9	7.7 ± 4.0	18.8 ± 4.0
3	36	41.8 ± 5.0	27.7 ± 2.3	6.9 ± 4.3	19.0 ± 4.3

The means are shown with standard deviations.

Since differential rejection of blood specimens in particular groups could lead to bias, the percentage of rejected specimens in each risk group was calculated for pre-menopausal women. For groups 0–3, the percentages were 30, 31, 27 and 31. In groups 0, 1 and 2 the reasons for rejection were similar but in the highest risk group significantly more blood samples were rejected because of irregular menstrual cycles or previous hysterectomies.

2. *Analytical methods*

Plasma oestradiol-17β was measured by radioimmunoassay using antisera to oestradiol conjugated through the 6 position to B.S.A. and used at a final dilution of 1:50,000. The basic assay technique was that described by Korenman *et al.* [5]. The coefficients of variation ranged from 16% at 10–50 pg/ml to 13% at 101–450 pg/ml. Recoveries averaged 78%. The sensitivity of the method was 6 pg/ml. Plasma progesterone was measured by the method of Swain [6].

RESULTS

1. *Plasma progesterone in pre-menopausal women*

The means of the logarithms of the plasma progesterone values are shown in Table 3. There are no apparent differences between the plasma concentrations in the two age groups 30–39 and 40–49. All statistical comparisons have therefore been carried out on the total groups.

There is a diminution in both the mean follicular phase and luteal phase progesterone levels with increasing risk. This is not significant in the follicular phase but in the luteal phase, the mean progesterone level is significantly lower in risk group 3 compared to that in risk group 0 ($t=2.18$: $P<0.05$).

The relation between declining progesterone levels and increasing risk of breast cancer is shown in Fig. 1. Levels of progesterone of less than 3 ng/ml in the luteal phase of the cycle have been taken to indicate anovulatory cycles. It can be seen that the proportion of such cycles in relation to ovulatory cycles increases as risk increases, and it is this that accounts for the difference between the mean levels. If only ovulatory cycles are considered, then there are no

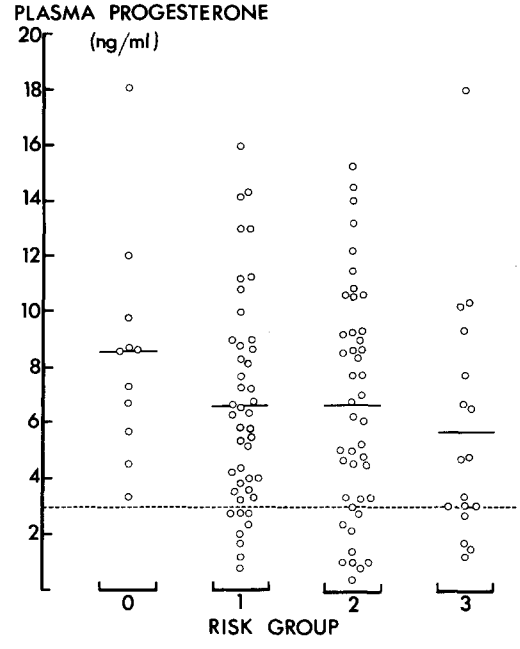

Fig. 1. Plasma progesterone values for women in the luteal phase, in risk groups 0–3. Points above the horizontal dashed line indicate presumed ovulatory cycles (more than 3 ng/ml). The solid horizontal bars show the arithmetic means.

Table 3. Means of logarithms of plasma progesterone levels in pre-menopausal risk groups

		Risk group 0	1	2	3
Follicular phase	All subjects	0.991 ± 0.345 (10)	1.059 ± 0.391 (39)	0.994 ± 0.406 (45)	0.952 ± 0.371 (19)
	30–39	0.844 ± 0.353 (6)	1.153 ± 0.291 (22)	1.230 ± 0.128 (19)	1.162 ± 0.157 (6)
	40–49	1.212 ± 0.206 (4)	0.938 ± 0.474 (17)	0.822 ± 0.453 (26)	0.856 ± 0.406 (13)
Luteal phase	All subjects	1.888 ± 0.202 (11)	1.740 ± 0.293 (45)	1.699 ± 0.401 (44)	1.644 ± 0.333 (17)
	30–39	1.889 ± 0.302 (5)	1.716 ± 0.258 (29)	1.794 ± 0.275 (20)	1.609 ± 0.239 (9)
	40–49	1.887 ± 0.090 (6)	1.783 ± 0.353 (16)	1.620 ± 0.473 (24)	1.684 ± 0.429 (8)

The results are expressed as the means of logarithms of the plasma progesterone values (ng/ml plasma) ± standard deviations.

Table 4. Mean of logarithms of plasma oestradiol levels in pre-menopausal risk groups

		Risk group 0	1	2	3
Follicular phase	All subjects	1.849±0.240 (11)	1.935±0.284 (35)	1.920±0.288 (45)	1.981±0.302 (19)
	30–39	1.769±0.170 (7)	1.999±0.253 (20)	1.920±0.304 (21)	2.005±0.390 (6)
	40–49	1.989±0.306 (4)	1.848±0.308 (15)	1.920±0.280 (24)	1.970±0.271 (13)
Luteal phase	All subjects	2.043±0.264 (12)	1.926±0.267 (44)	1.960±0.302 (43)	1.932±0.243 (17)
	30–39	2.138±0.236 (6)	1.968±0.277 (27)	1.951±0.292 (20)	1.886±0.235 (9)
	40–49	1.948±0.274 (6)	1.858±0.242 (17)	1.969±0.313 (23)	1.984±0.256 (8)

The results are expressed as the means of the logarithms of the plasma oestradiol values (pg/ml plasma × 10) ± standard deviation.

Table 5. Mean of logarithms of plasma progesterone and oestradiol levels in post-menopausal risk groups

	Risk group 0	1	2	3
Progesterone	1.075±0.324 (7)	1.182±0.333 (26)	1.170±0.422 (41)	1.136±0.402 (30)
Oestradiol	1.449±0.138 (7)	1.522±0.201 (42)	1.467±0.228 (43)	1.453±0.215 (33)

The units are as in Tables 4 and 5.

differences in progesterone levels in the four risk groups. The proportion of anovulatory cycles is 0, 18, 20 and 24% for risk groups 0–3 respectively. Since it is possible to ascribe an individual risk level to each woman (see Table 1), a regression of the logarithms of the progesterone concentration on risk level was calculated and found to be significant in the luteal phase ($r = -0.211$, $P < 0.05$, $n = 117$).

There is no significant association between risk and levels of progesterone in the follicular phase although the trend is in the same direction as in the luteal phase.

2. *Plasma oestradiol in pre-menopausal women*

When age groups 30–39 and 40–49 yr are taken together there are no significant differences between the means in the four risk groups in either the follicular or luteal phase of the menstrual cycle. In the age group 30–39 yr, however, the mean plasma oestradiol level is significantly greater in risk group 1 than in group 0 ($t = 2.686$, $P < 0.02$), but this effect is not duplicated in the 40–49 group.

There is no significant relationship between individual risk levels and plasma oestradiol levels. This result is unaltered if the data are re-analysed after removal of all anovulatory cases. The results are summarized in Table 4.

3. *Plasma progesterone and oestradiol in post-menopausal women*

The means of the logarithms of the progesterone and oestradiol values in post-menopausal women in the four risk groups are given in Table 5. There are no significant differences between the mean levels of these plasma steroids in the four groups. There is no significant correlation between individual risk levels, plasma progesterone or plasma oestradiol values.

4. *Relation of corpus luteal deficiency to single risk variables*

The design of this study was not appropriate for determining whether a single risk factor correlated with a particular endocrine abnormality. Nevertheless, an approximation may be obtained by calculat-

Table 6. Number of women with ovulatory or anovulatory cycles in association with given risk factors

Risk factor	Ovulatory	Anovulatory	(%)
Menarche before 14th birthday	54	21	(28)
Menarche, other	38	4	(10)
1st baby after 24th birthday	63	16	(20)
1st baby, other	29	9	(24)
Family history, positive	12	7	(37)
Family history, negative	80	18	(18)
Aetiocholanolone, less than 1 mg/24 hr	6	6	(50)
Aetiocholanolone, other	86	19	(18)

ing the number of anovulatory cycles which are associated with a given favourable or unfavourable factor, ignoring other factors that may be present. The results are shown in Table 6.

Anovulatory cycles are found more commonly in women with an early menarche, a family history of breast cancer, or a sub-normal excretion of aetiocholanolone, than in women with none of these factors, but only in the last instance is the distribution significantly abnormal ($X=6.5$, $P<0.02$).

The reality of the association between a sub-normal excretion of aetiocholanolone and anovulatory cycles is supported by the finding that in the entire population of pre-menopausal women (i.e. ignoring risk levels) there is a significant association between plasma progesterone levels in the luteal phase of the cycle and the excretion of aetiocholanolone in the urine ($r=0.231$, $P<0.02$, $n=117$).

This relationship is carried largely by risk groups 2 and 3 (which are characterized by a substantial proportion of women having a sub-normal excretion of aetiocholanolone). In risk group 2 the data are: $r=0.339$, $P<0.05$, $n=44$; and in risk group 3: $r=0.613$, $P<0.01$, $n=17$.

The relationship is not found in the follicular phase of the cycle or in post-menopausal women.

DISCUSSION

Sherman and Korenman [7] have made a persuasive case that inadequate corpus luteal function may provide an endocrine setting favourable for the development of mammary carcinoma. Our results support this view and indicate that increasing risk of breast cancer in pre-menopausal women is correlated with a diminished production of progesterone in the luteal phase of the menstrual cycle in the face of normal levels of oestradiol.

As far as other studies on high-risk groups are concerned, Sitruk-Ware *et al.* [8] have shown that women with benign breast disease have a significantly lower level of plasma progesterone in the luteal phase of the cycle compared with controls. Conversely, a prospective study carried out by the Royal College of General Practitioners shows that the incidence of benign breast disease diminishes as the content of progestagen in steroid contraceptives increases [9].

Once breast cancer is diagnosed, there is evidence that the same pattern of luteal insufficiency can be found. Grattarola [10] showed that only 17% of his patients with breast cancer had a secretory endometrial pattern indicative of ovulation, compared with 68% in normal women. Kodama *et al.* [11] have recently reported that Japanese patients with breast cancer have a sub-normal excretion of four steroids which vary with the phase of the menstrual cycle (one of which was pregnanediol). Swain and her colleagues found an increased incidence of luteal insufficiency in patients with advanced breast cancer but did not find this in patients with the early disease [12]. These results are not in agreement with those of the Manchester group [13, 14] who found raised levels of plasma oestradiol and progesterone in patients with benign and malignant breast disease. Their series did not apparently contain any women with anovulatory cycles, which is unexpected since these are common even in the normal population [15].

Anovulatory cycles or luteal insufficiency may not be the only critical factors associated with risk. There is at least an indication that early menarche is associated with luteal dysfunction (Table 6). A relationship between age at menarche and subsequent ovarian

function is implied by reports that women with a late menarche have a disproportionate incidence of amenorrhoea after using steroidal contraceptives [16].

Also, while a sub-normal excretion of androgen metabolites is a feature of the luteal abnormality in the high risk group, the androgen abnormality may not be a feature of anovulatory cycles in low risk groups. It may be that both factors contribute to high risk.

In the post-menopausal women in the Guernsey study, nothing remarkable was found. There may be a heavy bias in this group since the risk levels were calculated by Farewell [4] using data obtained from 48 women who subsequently developed breast cancer and these were predominantly pre-menopausal. It is not at all clear whether the risk factors used in the Farewell model would be useful in women well past the menopause.

The Guernsey prospective studies were originally designed to investigate the possible relationships between urinary and plasma hormones and the aetiology of breast cancer, but in the last few years the emphasis has shifted towards the identification of high-risk groups for periodic breast screening. The question therefore arises whether corpus luteal insufficiency would be a "useful" risk factor. One quite striking finding is that the women with a sub-normal excretion of urinary aetiocholanolone are predominantly those who, five years later, were shown to have a luteal deficiency. This implies that the abnormality is not sporadic but may occur in the majority of menstrual cycles in women at high risk. It also indicates that measurement of plasma progesterone and of urinary aetiocholanolone may give the same prognostic information but it may be possible to check this point when a sufficient number of the 5000 volunteers who gave a blood sample in 1965–1976 eventually develop breast cancer.

If a deficiency in the luteal phase secretion of progesterone does turn out to be an important determinant of risk, the correlation of such an abnormality might be possible.

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The Interaction of Hydrocortisone, Interferon and the Epstein–Barr Virus in Lymphoid Cells*

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Abstract—*Thirty-two human lymphoid cell lines of various origins were treated with hydrocortisone (HC) 20 μg/ml for* 3 days *and examined for cell viability, increase in the percentage of EBV antigen-positive cells, and interferon synthesis. Burkitt's lymphoma (BL) cell lines* (6/9) *under the effect of HC were characterized by a decrease in cell viability, increase in the percentage of EA antigen-positive cells, and poor interferon response in contrast to lymphoblastoid cell lines derived from the peripheral blood of normal individuals* (0/14) *or patients with infectious mononucleosis* (0/4). *The enhancement in the expression of EBV–EA as shown by the increase in the percentage of EA antigen-positive cells following HC was exclusively seen in BL cell lines* (6/9). *This effect could be blocked by phosphonoacetic acid (PAA). In contrast the effect on cell viability could not be inhibited by PAA but was abolished by pretreatment of the cells with interferon.*

INTRODUCTION

HYDROCORTISONE (HC) is used by clinicians for a variety of purposes. Its mechanism of action is complex and is still incompletely understood [1]. Hydrocortisone, like other steroid hormones, appears to control gene expression and protein synthesis following its binding to specific cytoplasmic receptors [2]. It can also affect lymphoid cell viability.

Human lymphoid cells are more corticosteroid-resistant than mouse lymphoid cells [3]. Differentiated cells are usually more resistant to corticosteroids than undifferentiated or blast cells [3]. Even though RNA and DNA synthesis have recently been shown to be inhibited by corticosteroids *in vitro* in both normal peripheral B and T human lymphocytes [4], mature lymphoid cells from the peripheral blood of normal individuals are generally corticosteroid-resistant, whereas leukemic lymphoblasts are more corticosteroid-sensitive [5]. Although the presence and relative number of receptors are not the only factors responsible for sensitivity to glucocorticoids [6, 7], it has been noted that there is a greater number of glucocorticoid receptors on human lymphoblasts, particularly null lymphoblasts, from lymphoblastic leukemia than on normal peripheral lymphocytes [8].

The Epstein–Barr virus is a Herpes virus with a very narrow host range. The human B lymphocyte is virtually the only cell known to support the *in vitro* replication of this virus. Its replication, even in the B lymphocyte, is usually restricted to the expression of an early nuclear antigen, EBNA. Occasionally, the expression of the virus genome can go on to the synthesis of another antigen, the early cytoplasmic antigen, EA, either spontaneously or following induction with IUdR or BUdR. Rarely does replication go on to the synthesis of the viral capsid antigen, or to the production of complete virus particles [9].

Like other Herpes viruses, the Epstein–Barr virus seems to persist in a latent state in the host following infection [10]. Under appropriate conditions, the culture of *peripheral blood lymphocytes* from a large percentage of EBV antibody positive individuals, will result in the establishment of permanent lymphoid cell lines containing the EB virus genome and expressing EBNA. The culture of the *Burkitt's lymphoma* cells will also yield permanent lymphoid cell lines positive for the EB virus genome and expressing EBNA. A few exceptional lymphoid cell lines either T or

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B will be free of EBV and EBNA negative [11, 12]. Reactivation of EBV as a result of immunosuppressive measures, or following administration of HC, might therefore occur and possibly lead to clinically apparent or significant disease. Strauch *et al.* [13] have reported the presence of EBV in the throat washings of approximately 50% of renal transplant patients under immunosuppression, suggesting reactivation of EBV since the prevalence of oral excretion in normal individuals is only approximately 10–20%. In a similar study we found a prevalence of 40% for EBV excretion in throat washings of renal transplant patients [14].

We have also reported that, *in vitro*, HC could adversely affect cell viability, with an enhancement of the expression of the EBV–EA and VCA antigens, and a delayed interferon response in 2 out of 6 lymphoblastoid cell lines containing the EBV genome [15]. These two cell lines were derived from BL cells. The four unresponsive cell lines were characterized by no altered cell viability, no enhancement of the expression of the EBV antigens, and a brisk interferon response. The purpose of the present paper is to summarize the results of studies done on 32 lymphoid cell lines of various origins showing different response patterns to HC according to the origin of the cell line tested.

MATERIALS AND METHODS

Leukocyte culture

Heparinized whole blood was separated on Ficoll–Hypaque (density 1.078) by centrifugation at 370 ***g*** for 20 min. The layer of lymphocytes thus obtained was resuspended in RPMI 1640 medium and washed by centrifuging the suspension at 370 ***g*** for 10 min. The suspension was washed twice and resuspended in RPMI 1640 medium containing 20% fetal calf serum. The concentration of cells per ml was adjusted to 10^6. One sample was then used for the HC test (test on fresh lymphocytes and another sample used to establish a lymphoblastoid cell line (spontaneous establishment). Cord blood cell lines were obtained from José Menezes. Establishment of these cell lines was obtained as described [11]. Lymphoma cell lines were obtained from various workers. All the cell lines were maintained in Falcon plastic bottles in suspension cultures with RPMI 1640 medium supplemented with 10–20% fetal calf serum and antibiotics readjusting the cell concentration to $2–3 \times 10^5$ with fresh medium twice a week.

The HC test

The fresh cells or the cell lines to be tested were divided into two aliquots before each experiment, one serving as control, the other being put in contact with 20 μg (4×01^{-5}M) of HC/ml (Solu cortef) for 3 days.

A dose–response curve had been done in our previous study [15]. This amount of HC produced a near maximal response for two of the parameters studied and a maximal response for the third, cell viability. It is approximately 100 times the physiologic concentration. Each experiment was done in triplicate.

The cell concentration used in all experiments was 10^6 cells/ml. The cells were incubated at 37°C and samples were taken at 1, 2 and 3 days after the addition of HC to do viable cell counts by the trypan blue dye exclusion method and to stain the cells for the EBV antigens by indirect immunofluorescence (IF) using an EA + VCA + serum, an EA − VCA + serum, and an EA − VCA − serum as control [16]. The titer of infectious virus in the supernatant of cultures with and without pretreatment of the cells with HC, was measured by the inhibition of Raji cell colony formation by EBV described by Rocchi *et al.* [17], as modified by Menezes *et al.* [11]. The supernatant of samples of 10^6 cells with and without HC was also examined by electron microscopy (EM). It was centrifuged at low speed to remove cell debris. The supernatant thus obtained was centrifuged at 80,000 ***g*** for 1 hr. The pellet was resuspended in acetate buffer and examined by EM after negative staining as described elsewhere [15]. Finally, the interferon titer in the whole culture after a 3-day incubation was measured by a micromethod previously reported [18], using Sindbis virus and human embryo fibroblasts. Control and test cultures were done in triplicate and each experiment was repeated twice.

Viral inhibitors

Phosphonoacetic acid (PAA), an inhibitor of Herpes virus DNA polymerase, was kindly supplied by Abbott Laboratories and it was used at the concentration of 50 μg/ml of RPMI 1640 culture medium. This concentration of PAA was shown in previous studies [19], to block EBV–DNA replication without being toxic to the cells. Exogenous interferon (10^6 units/ml) was a gift of Hans Strander and Kari Cantell. It was used, as in our previous studies [15], in amounts of 10^1–10^3 units/ml of RPMI 1640 culture medium. Cells were incubated with interferon for a period of 24 hr before the HC test was performed.

Table 1. *The effect of hydrocortisone* (20 μg/ml) *on lymphoblastoid cell lines after an incubation period of three days*

Group	Response pattern	Cell lines obtained from (N–PB), (CB), (BL–T)	Number of cell lines with Decrease in viability*	Increase in EA/VCA Ag's*	Detectable interferon titer* (units/0.1 ml)
I.	N–PB	Normal individuals (PB)	0/14	0/14	14/14 (36–384)
II.	N–PB	Mononucleosis patients (PB)	0/4	0/4	4/4 (16–96)
III.		Cord blood (CB)	3/5†	0/5	3/5 (6–960)
IV.	BL–T	Burkitt lymphoma tumor cells	6/9†	6/9‡	1/5 1/5: (192) 4/5: (<2)

N–PB: Peripheral blood–normal individuals.
BL–T: Burkitt lymphoma–tumor.
CB: Umbilical cord blood
EA: Epstein–Barr early antigen.
VCA: Epstein–Barr viral capsid antigen.

*Values obtained were an average of three samples.
†Decrease in viability significant at level $P<0.05$.
‡Increase in antigen production significant at level $P<0.05$.

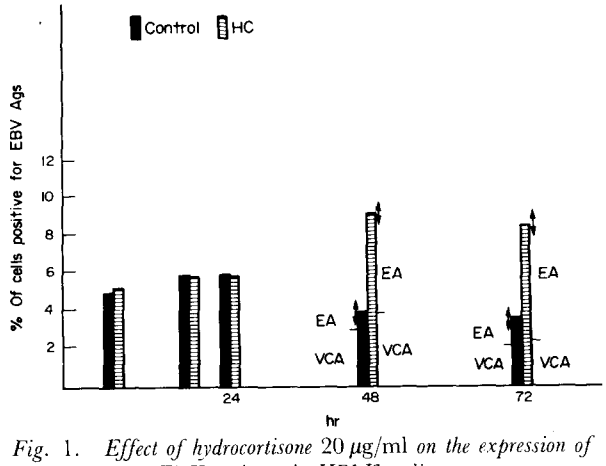

Fig. 1. *Effect of hydrocortisone* 20 μg/ml *on the expression of EBV antigens in HR1K-m line.*

RESULTS AND DISCUSSION

The overall results show that decrease in cell viability, enhanced expression of the viral genome, and interferon production can occur independently, following the addition of HC (Table 1 and Fig. 1).

Cell viability

Cell viability of EBV genome negative lines (BJA-B, a BL derived B lymphoid cell line, and MOLT 4, a leukemia derived T cell line), and of fresh lymphocytes from certain individuals (3 out of 5), could be decreased when treated with HC, suggesting a direct cellular effect of HC. More specifically, the percentage of E rosette-forming cells dropped significantly following HC treatment of the fresh lymphocytes from 58 to 33% ±2%.

Interferon

Interferon also could be induced by HC in fresh lymphocytes from certain individuals (3 out of 5 cases), suggesting that HC could have a direct effect on interferon production. Titers were usually less than 50 units/0.1 ml as compared to more than 100 units/0.1 ml following Sendai virus, another interferon inducer.

In the cell lines (Table 1) there seems to be an inverse correlation between the decrease in cell viability and the interferon response. When there is an effect on cell viability there is no interferon response and vice versa. We have also confirmed our previous finding [15] that exogenous interferon will prevent the adverse effect of HC on cell viability in HR1K. Similarly, addition of interferon (100 and 1000 units/ml) to a suspension of fresh lymphocytes treated with HC also prevented the effect of the hormone on cell viability. These three observations are probably different aspects of the same phenomenon. The significance of these unexpected findings are presently under study.

EBV antigens

In HR1K, an EBV producer BL-derived cell line, it was found that the antigen induced by the addition of HC was the early antigen (EA) (Fig. 1). There was an apparent increase of 1 $\log_{10}$ in virus titer. The addition of phosphonoacetic acid for 10 days prevented the effect of the hormone, added on the 7th day, on the expression of EBV–EA, but not on cell viability, thereby further dissociating the effect

of HC on the expression of the viral genome from its cellular effect. The number of cells on day 10 in the control and in the PAA treated samples rose to 3.1×10^6 cells $\pm 1.2 \times 10^5$ cells whereas, in the HC and HC+PAA treated samples it rose only to 2×10^6 cells $\pm 1.5 \times 10^5$ cells. The percentage of EA positive cells in the HC+PAA treated sample (2%), did not differ from that found in the control PAA treated sample (2.6%) but was significantly higher ($P<0.05$) in the HC treated sample (13%) showing that PAA could prevent the increase in EA positive cells caused by HC. In contrast, as noted also in our previous study [15], exogenous interferon (100 and 1000 units/ml) could not prevent this increase in EA positive cells although, as mentioned before, it prevented the adverse effect on cell viability, again dissociating the effect of HC on cell viability from its effect on the viral genome.

Response patterns to HC

The Burkitt's tumor cell lines were characterized by an absent or delayed interferon response, a decrease in cell viability and an increase in the number of EA positive cells following HC *in vitro* (BL-T pattern). In contrast, EBV–EA or EA–VCA positive cell lines derived from the peripheral blood (PB) of 14 normal individuals (N–PB pattern), 4 patients with infectious mononucleosis, and the EBV-transformed *marmoset* lymphoblastoid cell line B95-8 (not shown), a well-known EBV-producer cell line, did not show this decrease in viability, or increase in the percentage of EA-positive cells. However, all these LCL showed a brisk interferon response, following HC, when interferon was not already present in the culture (Table 1). A few exceptions to the BL–T pattern (3/9) were noted among the group of BL cell lines. We are now investigating the possibility that these cell lines were derived from "normal" lymphoid cells within the tumor mass rather than from the tumor cells themselves by doing karyotype analysis and studies of other markers of lymphoma cell lines as done by Nilsson and Ponten [12] who differentiated "lymphoma" cell lines as monoclonal and heteroploid among other characteristics from "lymphoblastoid" cell lines which were polyclonal and diploid. Alternatively, the lack of effect on viability by HC in 3 of the 9 BL cell lines could be the result of spontaneous or acquired resistance to HC, a phenomenon occasionally observed [7].

The EBV-transformed cord blood (CB) cell lines responded to HC by a decrease in cell viability (3/5) but no increase in the number of EA-positive cells in contrast to the responder BL cell lines, again showing that the effect of HC on cell viability can be dissociated from its effect on EBV-EA. It is interesting to note that HC does not have an enhancing effect on EA in the B95-8 cell line, a well-known EBV transformed and EBV-producer cell line, similar to HR1K in many respects, but derived from the peripheral blood of a normal marmoset in contrast to HR1K which is a lymphoma cell line derived from a Burkitt's tumor. The increase in the percentage of EA positive cells in lymphoma cell lines following HC may suggest a different control of the expression of EBV–EA in lymphoma derived cells, and could explain the characteristic EA antibody response of Burkitt's tumor patients. It is of interest in this respect that patients with ataxia telangiectasia, who share with Burkitt's lymphoma patients an increased susceptibility to developing lymphoma and a chromosome 14 anomaly, also show a high prevalence of EBV–EA antibodies [20]. Cell lines from the peripheral blood of these patients are being obtained and will be studied for their response to HC.

Finally, the different response pattern of lymphoma cells to HC noted in our *in vitro* study, in contrast to that of presumably normal cells, is generally consistent with the observations made by the other workers [3, 5, 6] and with the observed sensitivity of most lymphoid tumor cells *in vivo* to the lethal effect of glucocorticoids.

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Radiobiological Hypoxia of a Transplanted Rat Tumour and the Effect of Treatment with Cyclophosphamide*

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Abstract—*Isologous tumours, 8–10 mm in diameter, of the 37th–40th generation spontaneous adenocarcinoma LMC_1 have been grown subcutaneously in 150–200 g female Wistar rats and irradiated with ^{60}Co γ-rays before or 1, 4, 7 or 10 days after intraperitoneal injection of 150 mg/kg of cyclophosphamide (CP).*

Dose–response curves for delay in growth of the tumour given aerobic or hyperbaric (HBO) irradiation alone were biphasic and, above 15 Gy (AIR) and 20 Gy (HBO), their response was governed by a component of hypoxic cells. One day after CP the response of the tumour to aerobic irradiation was the same as when rendered hypoxic by clamping 15 min prior to treatment indicating that only hypoxic tumour cells survive in animals treated with drug. Three days later hypoxia is reduced by reoxygenation, but the hypoxic fraction is still significantly higher than before treatment with CP. Only by 7 and 10 days had the hypoxic fraction of clonogenic cells been reduced to a level characteristic of the untreated tumour.

Even though at 4 days after giving drug the hypoxic component of cells was significantly larger than normal for rats breathing air, no resistant component was found in the tumour response for HBO irradiation. This suggests that processes leading to the reoxygenation of a tumour after an initial treatment with drug may themselves permit an enhanced effect of subsequent HBO irradiation.

INTRODUCTION

THE USE of cytotoxic drugs as adjuvants to radiotherapy offers a potential therapeutic advantage in improved control of primary tumours. Thus doses of cytotoxic agents tolerated by the host may be used to kill a large proportion of cells in the tumour prior to irradiation [1, 2]. However, many such agents are less effective against non-proliferating than against cycling populations of cells [3]. Some at least of the non-proliferating cells in solid tumours may also be hypoxic [4], so that unless reoxygenation occurs in the conditions prevailing after drug treatment, subsequent irradiation will have a less than optimal effect. We have examined the response of an experimental tumour whilst subjected to elevated, normal or reduced conditions of oxygenation, to irradiation alone or after pre-treatment by the cycle-active alkylating agent cyclophosphamide (CP). This tumour contains a clonogenic cell population relatively resistant to the action of the drug [2] and has a hypoxic fraction, measured by cell assay *in vitro* of approximately 18% [5]. The present experiments employed the endpoint of delay in the macroscopic growth of the tumour [6].

MATERIALS AND METHODS

Tumours of the 37th–40th transplant generations of the rat adenocarcinoma LMC_1 [7] were implanted subcutaneously in the flank of female 150–200 g Wistar rats of John's strain, using the method of Thomlinson [8]. Animals were assigned randomly to treatment groups when their tumours first reached a mean diameter of 8–10 mm, as determined by ca-

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liper measurements. After treatment they were caged in groups of 6, allowed food and water *ad libitum* and their tumours measured daily.

Cyclophosphamide ('Endoxana'; Ward Blenkinsop, Bracknell) was injected intraperitoneally at a standard dose of 150 mg/kg of body weight. This dose reduces the surviving fraction of clonogenic cells in the tumour to about 3% [2] and also results in a less than two-fold change in tumour volume over the first 10 days after treatment [5].

Rats were immobilised for irradiation by intraperitoneal injection of 65 mg/kg of sodium amylobarbitone ('Amytal'; Lilly, Basingstoke) and immediately after treatment were given 0.8 mg/kg of 'Megimide' (Nicholas, Slough) to facilitate recovery from the hypnotic. Tumours were irradiated locally with ^{60}Co γ-rays at a dose-rate of approximately 1.90 Gy/min, the rats lying within a pressure chamber [6]. When treated with the rats breathing hyperbaric oxygen (HBO, 4 At abs) or air, the tumour lay unsupported in the irradiation beam. Tumours were made artificially hypoxic by occluding the blood supply to the tumour with a 'U' shaped clamp applied 15 min prior to irradiation of rats breathing air. All irradiations were given as single doses. In rats treated first with CP, irradiation of the tumour in air or under hypoxia was carried out 1, 4, 7 or 10 days later. Irradiation in HBO was also used at 4 days after CP treatment.

After treatment the time taken for each 8–10 mm tumour to grow to an average diameter of 25 mm was measured. The difference in mean times taken by tumours in treated and untreated groups of rats constitutes the growth delay. The *rates* of regrowth of tumours treated by irradiation alone and those in rats given CP before irradiation has previously been shown to be the same [5].

RESULTS

After irradiation alone, the growth delay for tumours treated when clamped increased progressively with dose, while the growth delay data for tumours irradiated in rats breathing air or HBO were biphasic (Fig. 1). Data for doses up to and including 20 Gy (HBO) and 15 Gy (air) could be fitted by redrawing the dose response curve for irradiation under hypoxic conditions assuming oxygen enhancement ratios of 3.0 and 2.7 respectively (curves A and B, Fig. 1). With larger doses given in either condition, delay data were consistent with the response curve for hypoxic tumours

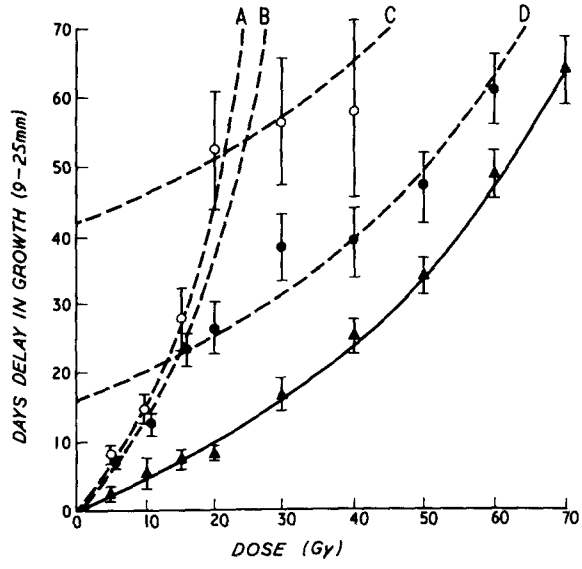

Fig. 1. Radiation dose–response for LMC$_I$ in rats before treatment with CP. ▲ hypoxic; ● aerobic; ○ HBO. Mean delays ±2 S.E., 4–6 rats per treatment group. Hypoxic curve fitted free-hand, curves A–D fitted as described in text.

when this was displaced vertically upward, to an extent greater for irradiation in HBO (Curve C, Fig. 1) than in air (Curve D, Fig. 1).

The delay in growth of LMC$_I$ tumours following injection of rats with 150 mg/kg CP alone was 12.8 ± 0.3 days. When this mean value was subtracted from the total delay obtained for tumours irradiated when clamped, but 1, 4, 7 or 10 days after treatment of the rats with drug, the delays due to the irradiation did not differ significantly from those for hypoxic tumours in rats not given CP (Fig. 2). After CP and with irradiation

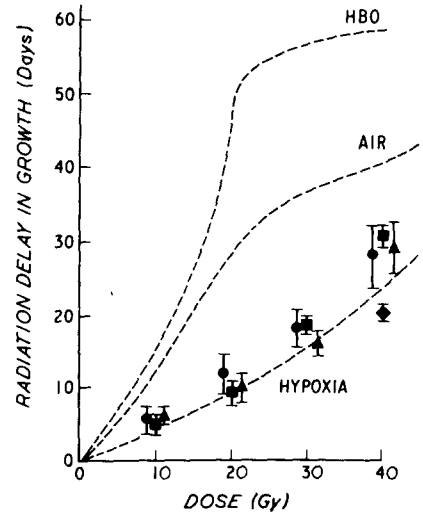

Fig. 2. Dose–response for LMC$_I$ irradiated under hypoxic conditions after treatment of rats with CP, ◆ 1 day; ● 4 days; ■ 7 days; or ▲ 10 days previously. Five to six rats per treatment group. Mean delays ±2 S.E.; dashed curves are free-hand fits to means of experimental data given in Fig. 1.

given in *aerobic* conditions at day 1, the growth delay of the tumours due to irradiation was also consistent with the radiation dose response curve for *hypoxic* tumours in rats not previously treated with drug (Fig. 3).

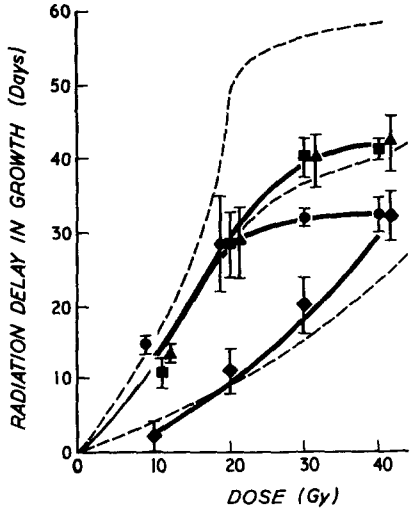

Fig. 3. Dose–response for LMC_I irradiated in air after treatment of rats with CP: ◆ 1 day; ● 4 days; ■ 7 days *or* ▲ 10 days *previously. Five to six rats per treatment group. Mean delays* ±2 S.E. *Dashed curves as in Fig. 2.*

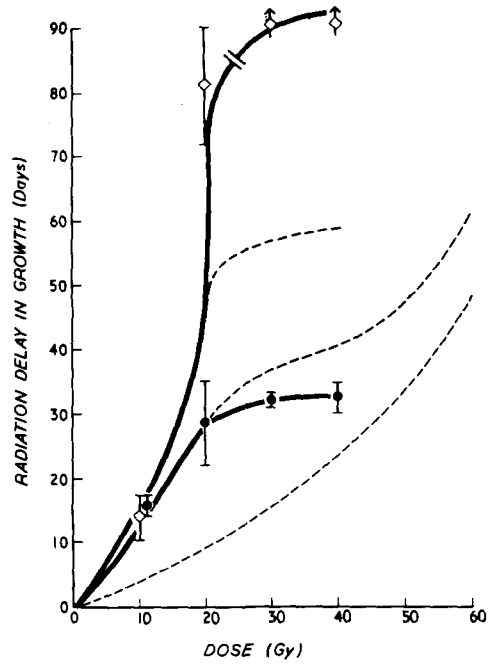

Fig. 4. Dose–response for LMC_I irradiated in, ● *air or* ◇ *HBO,* 4 days *after treatment of rats with CP. Five to six rats per treatment group, mean delays* ±2 S.E. *Dashed curves as in Figs. 2 and 3.*

However, 4 days after CP, a biphasic curve for irradiation in air was restored but the delays due to 30 and 40 Gy remained significantly less than the corresponding delays for aerobic irradiation alone ($P>0.01$). By 7 and 10 days after CP, the tumour response to all doses of aerobic irradiation was the same as for tumours in rats that had not been treated previously with drug.*

When tumours were irradiated in untreated hosts breathing HBO, a residual resistant component was revealed at high dose (Fig. 1). Four days after CP when the resistant component in air breathing rats is even higher than normal, this residual component could not be shown in the radiation response of tumours when treated in HBO, none having yet regrown after 30 or 40 Gy more than 150 days after treatment (Fig. 4).

DISCUSSION

The shapes and positions of curves for delay in tumour growth (Figs. 1–4) are believed to be determined mainly by the relative sensitivities of clonogenic cells within a population, rather than the absolute size of that population. Thus Thomlinson [10] found that for the Fibrosarcoma RIB_5 40 min after 35 Gy with the tumour clamped (for RIB_5C reducing the clonogenic cell population by approximately 10^3 [11]) the delay data obtained for second doses given in air were changed little from that for aerobic irradiation of previously untreated tumours. We have found also that the delay curves for LMC_I tumours irradiated at 5 mm diameter either clamped or in air, (Moore, unpublished) resemble closely those treated at 9±1 mm (Fig. 1). It seems valid then, to determine changes in the proportion of hypoxic cells by shifts in the growth delay curves for tumours even though the total number of cells may alter with time after an initial treatment, as occurs for LMC_I in the days following injection of CP [2]. Thus for rats treated with CP and where all the clonogenic cells within the tumour had been made uniformly resistant to radiation by clamping, little change was encountered in the position and shape of the dose–response curve for growth delay determined at any of the chosen intervals after treatment with drug.

The delay data for tumours irradiated under aerobic conditions does however reveal differences dependent upon the time elapsed after giving drug. Notably, at 1 day after CP the response to radiation was almost indistinguishable from that for a tumour in which

*Radiation doses greater than 40 Gy were in general not given to CP treated animals since the combined delay in tumour growth permitted an increased incidence and more widespread development of metastases [9].

all cells had been made hypoxic by clamping (Fig. 3). A similar shift has been observed for the tumour RIB_5 when second doses of radiation were given 40 min after a first dose sufficient to destroy all the well-oxygenated cells [10]. However for LMC_I the effect persisted much longer; the data indicated that the proportion of hypoxic cells had still not been reduced by reoxygenation to pretreatment value even 4 days after CP. That alkylating agents may preferentially spare hypoxic clonogenic cells has been shown recently for the KHT sarcoma treated with nitrogen mustard [1]. The phenomenon however, is not universal; the proportion of hypoxic cells in the B16 melanoma was unchanged when assayed 2 hr after CP [12]. In the cases where the proportion of hypoxic cells among survivors is raised, as for LMC_I, the rate of reoxygenation may vary between tumour types, as it does after a dose of radiation [13]. The LMC_I tumour has a relatively low Cell Loss Factor of 0.3 [7] and after 150 mg/kg CP but before regrowth occurs, its volume never regresses below that on the day of treatment [2]. It is unlikely then, that the type of reoxygenation seen for the C_3H mammary carcinoma which is rapid and complete [12], would occur in LMC_I, which in this respect more closely resembles the very slowly reoxygenating osteosarcoma described by van Putten [14].

In the light of these observations, the extent to which HBO enhanced the effect of the larger doses of radiation given 4 days after CP, is puzzling (Fig. 4). In rats not treated with CP, irradiation in HBO revealed a residual proportion of hypoxic cells (Fig. 1). That reduced competition for oxygen from sterilized tumour cells in rats treated with CP would enable this hypoxic component to reoxygenate is reasonable but the same argument holds for tumours in rats breathing air where the hypoxic component is larger than normal four days after CP (Fig. 4). However at this time reoxygenation is still in progress and normal levels of tumour hypoxia are not attained until 7–10 days (Fig. 3). Thus, the situation prevailing after treatment with CP is in contrast to that before, when the oxygen concentration of critical cells is decreasing and hypoxia is a transient state leading to cell death and necrosis. On this basis, factors operating in favour of reoxygenation of the tumour after CP treatment also may favour the more efficient oxygenation of erstwhile hypoxic cells during irradiation in HBO.

Apart from this apparent enhanced effect of HBO irradiation 4 days after CP, which requires further studies at other time intervals, the results of these experiments clearly indicate that the systemic use of drug before local radiotherapy under aerobic conditions may either prejudice local control of the tumour (i.e. at 1 and 4 day intervals for LMC_I) or at best (7–10 days) have little or no synergistic effect. From the clinical standpoint the therapeutic optimum, i.e. a reduction in the hypoxic fraction below a level characteristic of the untreated tumour, was not achieved. If this depends ultimately upon a reduction of tumour volume and a diminution of inter-capillary distances this is not achievable with LMC_I using single doses of other chemotherapeutic agents e.g. Methotrexate, Thiotepa and Adriamycin (Dixon, unpublished). Doses of CP greater than 150 mg/kg result in an unacceptable mortality, and though this may be avoided by the use of a split dose, actual regression of the LMC_I tumour does not occur [15].

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Breast Cancer: The Error of the Exponential

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Abstract—*Survival curves for patients with breast cancer have been studied. A significant deviation from the exponential was found, and it has been shown instead that a log normal type of distribution of survival times is responsible for the shape of the survival curve. Conclusions drawn from acceptance of the exponential are therefore in error, whereas conclusions drawn from the log normal type of distribution are compatible with clinical observations and lead to the acceptance of the concept of biological predeterminism.*

INTRODUCTION

FASHIONABLE views concerning the behaviour of breast cancer emanate from the the demonstration of an apparent exponential survival curve for those patients dying from their disease [1]. It is said that this indicates that the process of dying from carcinoma of the breast is random, that the probability of dying from cancer is constant and that the number of patients dying during equal periods is a fixed proportion of those that were alive at the beginning of the period.

It would follow from this hypothesis that breast cancer is incurable in the sense that the last cancer cell cannot be eliminated from the body. This signifies that all breast cancers metastasise and that these metastases are held in a dormant state by general or local tissue immunological control. Breakdown of immunity then occurs as a random event and the metastases then grow and eventually cause the death of the patient. However, it is postulated that temporary control of the tumour may be achieved by hormone or chemotherapy administration and survival therefore prolonged [2].

However, Boag [3] investigated the distribution of survival times for patients dying from cancer at several sites. He found a continuous unimodal distribution with a skew to the right. A logarithmic transformation of the variate made the distribution approximately normal. He therefore described this as being of log normal distribution. Data collected from a number of different centres all yielded histograms that were essentially similar in form. He considered that this indicated that if the treatment had not been successful in eradicating the tumour completely, the length of survival must be dependent principally on the growth rate of the tumour remnants and not upon the form of treatment used. Boag also concluded that a log normal distribution indicated that a proportion of patients were cured of their disease, since at the end of 10 yr a group of patients were still alive and symptom free, and the group was too large to be fitted into the tail of the log normal distribution. However, a short follow up time left the distribution of survival times for breast cancer patients in some doubt.

Although the exponential survival curve and that obtained from the log normal distribution of survival times are similar in appearance when drawn on a semi log scale, they are in fact separate, statistically identifiable curves. Different, irreconcilable concepts of the natural history of breast cancer stem from the acceptance of each of these curves. In this paper an attempt has been made to identify the correct form of the survival curve for breast cancer patients.

MATERIALS AND METHODS

An investigation has been made into the distribution of survival times of 363 patients dying from breast cancer. These were women who presented with disease confined to the breast or associated with mobile homolateral axillary lymph nodes such that it was possible

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to remove all macroscopic disease by simple or radical mastectomy. None of the patients had clinical evidence of distant metastases at presentation, but investigations were limited to a chest X-ray and an X-ray to exclude bone metastases if pain was present. The 363 patients comprised the sum total registered at the Christie Hospital during 1955 who conformed to these criteria. Survival has been calculated from the time of the initial surgical operation.

RESULTS

The distribution of survival times (Fig. 1) was found to be unimodal with a skew to the right, similar to that found by Boag [3]. When a semi log plot of the survival curve is made (Fig. 2), it is found that there is a shoulder corresponding to the first 12 months. Campos [1] has suggested that the shoulder represents the time required for a tumour to reach a size that will kill the host and that the curve becomes exponential after this. When the author's curve is inspected with the first 12 months excluded, it is found that there is a tendency for the curve to be convex downwards. However, this deviation from the exponential is not statistically significant.

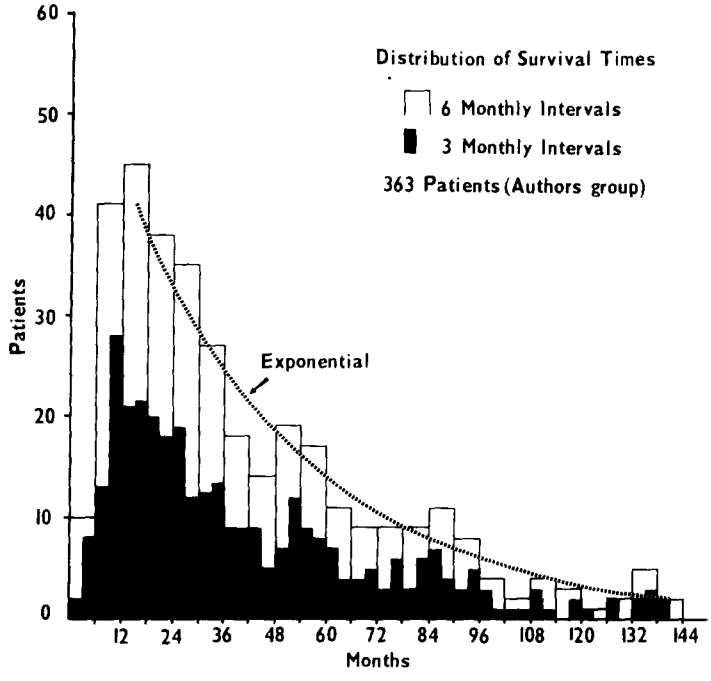

Fig. 1. Histogram showing distribution of survival times (author's group of patients).

DISCUSSION

Although the author's curve with the first 12 months excluded showed no significant deviation from the exponential, the question must be asked: Is this part of the curve truly exponential, or is the curve not exponential and are there too few patients to make the deviation statistically significant? In an attempt to answer this question a survival curve, obtained from the literature for 4304 breast cancer patients, has been studied [4] (Fig. 2). This deviates from the exponential in a similar manner to the curve derived from the author's group of patients and the deviation is statistically significant (Kolmogorov–Smirnov Test $P \ll 0.001$). However, if the patients in this large published series are not homogeneous, the underlying survival distribution may still be exponential. The group has therefore been divided into those having histologically positive and those having negative axillary nodes. The survival curves for both subgroups are a very poor fit to the exponential ($P \ll 0.001$).

There is, therefore, no doubt that the survival curve for breast cancer patients has been mistakenly identified as being of an exponential form. The reason for this is that when the curve is derived from a small number of patients the deviation from the exponential

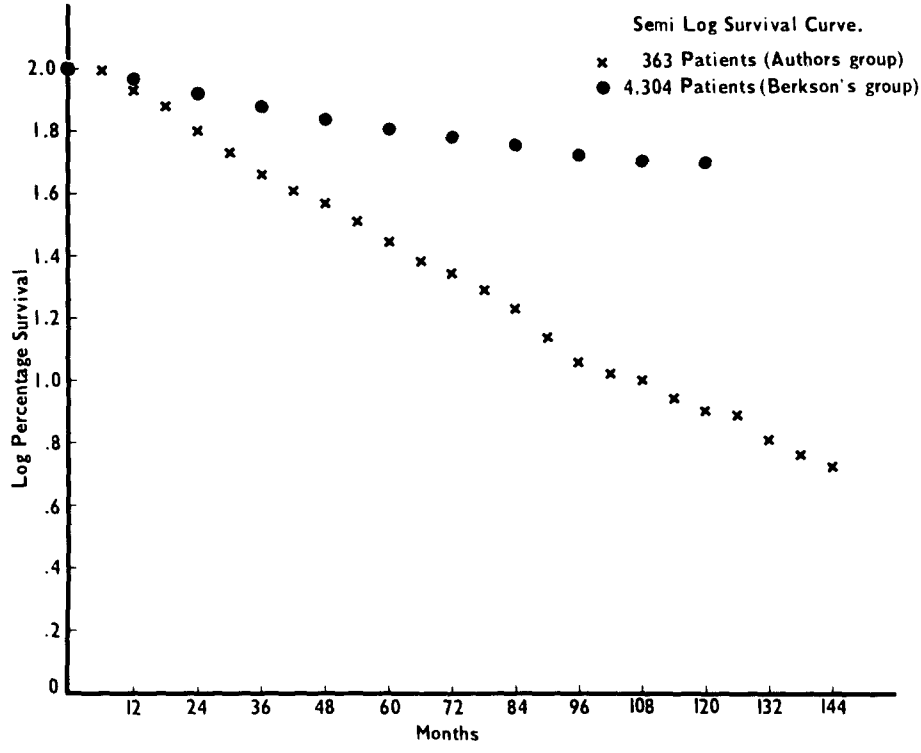

Fig. 2. Semi log survival curve (author's and Berkson's group of patients).

will not reach significant values. When a curve derived from large numbers of patients is considered, a statistically significant deviation from the exponential becomes apparent. The form of the survival curve must result instead from a continuous unimodal distribution of survival times with a skew to the right, similar to that described by Boag. However, when large numbers of patients dying from cancer at various sites are considered, there is a significant deviation from the log normal [5], and it is probable that the distribution does not accurately correspond to any specific statistical model. Therefore, for the rest of this article, the term log normal type will be used when referring to the observed distribution.

The rest of this discussion is hypothesis regarding the significance of the error of the exponential. Since the observed distribution approximates to the log normal model. Boag's conclusions are considered.

The validity of Boag's contention that the length of survival must be dependent principally on the growth rate of the tumour is first investigated. It has been shown that cancers grow in most cases for most of the time in an exponential manner, and that death occurs at approximately the 45th doubling, when a critical mass of tumour is present [6–9]. It would therefore be expected that a relationship exists between growth rate and duration of survival. Four papers published in the literature confirm this.

1 and 2. Tumour growth rate and duration of survival are directly proportional [10, 11].

3. There is a log normal type of distribution both of survival and of tumour growth rates [12].

4. When tumours are induced in mice using ultra-violet light, the intervals required for the cancers to appear formed a log normal type of frequency distribution [13].

It is thus apparent that the log normal type of distribution of survival times has been produced by a log normal type of distribution of tumour growth rates, and therefore that survival in those patients not cured depends principally on the growth rate of the tumour.

Boag's conclusion that a cured group of patients can be identified statistically is now considered. If this conclusion was valid this would indicate that there is a group of patients with breast cancers which fails to metastasise. There are three observations which appear to support this concept.

1. If a survival curve is drawn for a group of patients suffering from breast cancer, after 15 yr, the curve is almost parallel to that constructed for a healthy group of women of similar age distribution. This indicates that most patients with breast cancer are cured if they have survived 15 yr [14].

2. A constant metastatic pattern is found in

unselected groups of breast cancer patients [15–17]. This implies a random distribution of clinically observed metastases within the limitations of constant factors in individual patients. This must result from a random distribution, implantation and growth of metastasising tumour fragments within the limitations of constant mechanical factors and is incompatible with the concept of intermittent or selective immunological control. Therefore, if metastases fail to appear clinically, the primary tumour has failed to metastasise.

3. When ^{51}Cr labelled cancer cells were injected into mice [18], it was found that the cells lodged in the organs so as to produce the subsequently observed metastatic pattern. It was also found that the cells passed rapidly through the organs which were rarely the site of metastases. Most of the cancer cells eventually died in transit in the blood stream before lodging in any organ and were finally broken down in the liver and spleen. This observation implies that when cancer cells lodge in organs a constant proportion of them will give rise to clinically apparent metastases. Therefore, if clinical metastases fail to appear the primary tumour has failed to produce metastasising cells capable of implantation in the tissues or alternatively has done so in insufficient numbers to produce clinical metastases.

It has been shown that when metastases develop they have almost all been initiated during the preclinical phase of the parent tumour [10]. There thus seems little doubt, that the conclusions drawn from the log normal type of distribution, that some patients with breast cancer are cured, may be explained by postulating that some cancers do not shed their cells or alternatively shed very few cells into the blood stream.

The log normal type of distribution of survival times is produced by a log normal type of distribution of tumour growth rates. Therefore, patients who have tumours which metastasise may have slowly or rapidly growing tumours. It may therefore be expected that there is no correlation between metastatic potential and tumour growth rate [19]. The validity of this supposition is now examined.

In a previous paper by the author [17] it was demonstrated that patients with a long latent interval had slowly growing tumours. These must represent the extreme end of the skew of a log normal type distribution. However, most of the patients developed widespread metastases and died from their disease.

Patients with poorly differentiated tumours have lower survival rates than those with better differentiated tumours [4]. It is pertinent to enquire whether this is because poorly differentiated tumours have a high metastatic potential or because they have a faster growth rate. Four observations provide the answer to this question.

1. When a group of breast cancer patients having a long latent interval was assessed, it was found that anaplastic tumours did not differ in growth rate from better differentiated tumours [17].

2. It has been shown that the histological pattern is similar whether a group of tumours show clinical evidence of metastases after 1–2 yr or after a latent interval of 5 yr [15].

3. It has been demonstrated that patients with anaplastic tumours develop local recurrence twice as frequently as those with better differentiated tumours [20].

4. Tumour doubling time is not related to the degree of tumour differentiation [8].

There is, therefore, little doubt that the degree of tumour differentiation is an indication not of tumour growth rate but of metastatic potential.

It has been reported [21] that a characteristic cellular morphology can be identified in tumours recurring more than 10 years after the initial treatment, i.e. slowly growing tumours [16]. On the basis of this a typing system has been devised which does not correlate with other histological classifications but shows good correlation with survival. This typing system would therefore appear to give an indication of tumour growth rate.

CONCLUSION

Acceptance of the exponential function of the survival curve has led to a misunderstanding of the natural history of breast cancer. A realisation of the log normal type of distribution leads us to the acceptance of the concept of biological predeterminism [22]. When the first cancer cell forms the outcome of the disease is determined. Non-metastatic breast cancer is curable. When metastases have occurred, there is no evidence that host immunity or treatment significantly alters survival.

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VM26 (NSC-122819): A Clinical Study in Advanced Cancer of the Lung and Ovary*

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Abstract—*Fifty-five evaluable patients with advanced carcinoma of the lung* (25 *squamous*, 19 *adeno*, 11 *small cell*) *and* 16 *patients with advanced adenocarcinoma of the ovary were treated with VM*26 *in a phase* II *study. Only one response was noted, a partial* (>50%) *remission in squamous cell cancer of the lung for* 8+*months. The drug was well tolerated with minimal nausea, vomiting, anorexia and alopecia. Myelosuppression was infrequent in patients not previously treated with chemotherapy and usually tolerable in patients previously treated. Orthostatic hypotension, hepatotoxicity and peripheral neuropathy were not observed. On the basis of this report VM*26 *does not appear to demonstrate any significant antitumor effect in advanced cancer of the lung and ovary.*

INTRODUCTION

PODOPHYLLOTOXIN derivatives have been studied since the late 1940's for evidence of clinical antitumor activity. Two new semisynthetic derivatives have been recently developed by Sandoz Laboratories: VM26, 4′-demethyl 1-0-[4,6-0-0(2-thenylidene)-β-D-glucopyranosyl] epipodophyllotoxin (NSC-122819), and VP16-213, 4′-demethyl-1-0[4,6-0(ethylidene)-β-D-glucopyransosyl] epipodophyllotoxin (NSC-141540).

In cell cultures exposed to VM26 and VP16-213 metaphase arrest has been shown, but unlike the parent compound podophyllotoxin, microtubular alterations have not been demonstrated. A premitotic block preventing entry of cells into mitosis appears to be the predominant mechanism of action [1, 2, 3]. Additionally, these drugs cause inhibition of thymidine incorporation [4, 5, 6]. Both compounds are active in Ehrlich ascites, Walker carcinosarcoma, Sarcoma 37 and 180, and several murine leukemias including L1210 [7, 8, 9]. Both drugs demonstrate schedule dependency in the latter system.

VP16-213 has undergone more extensive clinical evaluation [10]. Antitumor activity has been observed in acute nonlymphocytic leukemia, Hodgkin's and non-Hodgkin's lymphoma, ovarian carcinoma and small cell carcinoma of the lung [11–18]. VM26 has been studied by the European Organization for Research of Treatment of Cancer (E.O.R.T.C.) in various solid tumors and responses were noted in Hodgkin's and non-Hodgkin's lymphoma, bladder and breast carcinoma [19]. Other tumor types were not represented by a sufficient number of patients to assess drug activity. Another report documents responses in intracranial neoplasms [20].

We initiated a broad phase II study of VM26 in various solid tumors in 1976 and our results in lung and ovarian cancer are the subjects of this report.

MATERIALS AND METHODS

Patient eligibility

All patients entered on the study had histologically proven advanced squamous cell, small cell undifferentiated (oat cell) or adenocarcinoma of the lung, or adenocarcinoma of

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the ovary. The patients were informed as to the investigational nature of the study and all gave written consent in accordance with our institutional policy. There were no age restrictions. Other criteria for eligibility included the following: a life expectancy of at least 6 weeks; no prior chemotherapy with a podophyllotoxin derivative; measurable disease outside a concurrently or previously irradiated site; initial white blood cell count $>3000/mm^3$ and platelet count $>100{,}000/mm^3$; adequate renal function as defined by a serum creatinine $\leqq 1.5$ mg% and BUN $\leqq 25$ mg%; adequate liver function as defined by a SGOT < twice upper limits of normal and total bilirubin <2.0 mg%; and no previous chemotherapy within the past 28 days and evidence of hematologic recovery from that therapy.

On study evaluation

Patients were evaluated while receiving therapy with serial CBCs, chest roentgenograms, liver and renal function tests and tumor measurement. A two week survival on therapy constituted an adequate trial.

Treatment protocol

VM26, supplied by the National Cancer Institute, Bethesda, Maryland, U.S.A. in 10 mg/ml, 5 ml ampoules, was diluted in at least 5 vol of sodium chloride injection U.S.P. or 5% dextrose in water and administered in an established IV line over 30 min to 1 hr. Good risk patients received an initial dose of $30\ mg/m^2$ daily × 5 every 3–4 weeks while poor risk patients with prior extensive radiation therapy and/or chemotherapy were started at $20\ mg/m^2$ daily × 5 every 3–4 weeks. Subsequent doses of VM26 were adjusted to nadir blood counts and patient tolerance. Dose escalation was encouraged in the absence of myelotoxicity.

Definitions of response

Complete remission was defined as the disappearance of all clinical evidence of active tumor for a minimum of 4 weeks together with a disappearance of all tumor-related symptoms. Partial remission was defined as >50% decrease in the sum of the products of the largest perpendicular diameters of all measured lesions lasting at least 4 weeks in the absence of significant increase in any one lesion or the appearance of new lesions. Progressive disease was defined as unequivocal increase of any measured lesion or the appearance of new lesions.

Patients. Seventy-seven patients (37 men, 40 women) were entered onto the study. Six patients were considered early death; that is, they died within 14 days of initiation of therapy from tumor-related causes and were inevaluable for toxicity or response. Additional patient characteristics with respect to age, Karnofsky performance status, and the nature of prior treatment are outlined in Table 1.

Table 1. Patient characteristics

Tumor	No. of pts entered	Male	Female	No. of pts evaluable	Age (mean, range)	Karnofsky performance status (mean, range)	Prior therapy: Chemotherapy	Prior therapy: Radiotherapy	Prior therapy: Both	Prior therapy: None
Lung										
Squamous cell carcinoma	28	21	7	25	60, 35–78	68, 40–90	1	13	6	8
Adenocarcinoma	19	9	10	19	57, 41–80	63, 40–90	3	2	11	3
Small (oat cell)	14	7	7	11	57, 42–78	67, 40–80	4	0	10	0
Ovary, adenocarcinoma	16		16	16	58, 40–75	71, 40–90	12	0	4	0
Totals	77	37	40	71						

RESULTS

Antitumor activity

Of the 71 evaluable patients in all tumor categories only one partial response was observed in a patient with squamous cell carcinoma of the lung. The duration of this response was 8+ months. Of significance was the lack of response in small cell carcinoma of the lung and adenocarcinoma of the ovary.

Toxicity

Non-hematologic toxicity could be evaluated in 71 patients and consisted of nausea in 8% of patients, vomiting in 5% and alopecia in 7%. Nausea and vomiting were consistently mild to moderate in nature and easily controlled with anti-emetics. Peripheral neuropathy, hepatoxocity and hypotension were not observed.

Hematologic toxicity and days to nadir by dose level through three course (mean number of courses of therapy per patient=3) is described in Tables 2 and 3, separated by the presence or absence of prior chemotherapy. There did not appear to be any cumulative myelosuppression nor any time delay in administering subsequent courses of therapy. Significantly more leukopenia (wbc $<3000/mm^3$ in 27% of evaluable courses) and thrombocytopenia (platelets $<100,000/mm^3$ in 18% of evaluable courses) was observed in those patients who had received prior chemotherapy ($P<0.01$). There was one drug-related death from sepsis in a

Table 2. Hematologic toxicity. Patients with prior chemotherapy

Dose level ($mg/m^2 \times 5$)	Course	No. of pts.	Median nadir wbc ($\times 10^3/mm^3$)	Range	Median days to nadir	Median nadir platelets ($\times 10^3/mm^3$)	Range	Median days to nadir
20	1	19	5.4	2.6–8.4	15	232	64–384	14
	2	6	4.8	2.7–7.8	21	210	150–380	21
	3	3	4.0	3.6–6.1	21	174	172–176	21
30	1	18	4.0	1.2–7.3	18	170	65–414	13
	2	3	3.0	2.0–7.0	17.5	202	160–244	6
	3	2	4.9	3.5–6.3	24	211	166–255	—
40	1	11	2.7	0.17–5.5	13	188	12–370	14
	2	3	2.9	0.3–3.1	11	198	4–271	14
	3	2	3.1	2.8–3.5	20.5	122	—	11
50	1	2	3.9	2.4–5.5	11.5	141	84–198	6
	2	2	3.5	3.5–3.6	7	139	130–149	7
	3	1	2.7	—	3	188	—	

Table 3. Hematologic toxicity Patients without prior chemotherapy

Dose level ($mg/m^2 \times 5$)	Course	No. of pts	Median nadir wbc ($\times 10^3/mm^3$)	Range	Median days to nadir	Median nadir platelets ($\times 10^3/mm^3$)	Range	Median days to nadir
20	1	3	5.5	2.6–9.8	13	230	200–280	8
	2	1	7.4	—	—	330	—	—
30	1	20	5.0	2.3–8.3	18	265	90–502	13.5
	2	12	5.7	1.3–7.8	14	319	98–442	14
	3	5	5.5	3.3–6.6	16	268	216–600	14
40	1	10	4.3	0.4–7.8	13.5	285	20–525	14
	2	6	4.6	2.9–5.9	14	415	235–514	14
	3	4	5.1	2.8–8.2	14	507	182–600	10.5
50	1	1	5.6	—	14	182	—	14
	2	1	4.8	—	14	184	—	14
	3	1	5.5	—	7	210	—	14

patient with small cell carcinoma of the lung whose white blood cell count was 0.17 $\times 10^3/mm^3$ and platelet count 26,000/mm^3 at the time of his demise.

DISCUSSION

VM26 was administered to 55 evaluable patients with advanced carcinoma of the lung (25 squamous, 19 adenocarcinoma, 11 small cell) and to 16 patients with ovarian carcinoma.

Only one objective response was observed in squamous cell carcinoma of the lung.

The drug appeared to be well tolerated with minimal nausea and vomiting and tolerable myelotoxicity.

The lack of response is disappointing particularly in small cell carcinoma of the lung and adenocarcinoma of the ovary where VP16-213, an analog of VM26, has shown evidence of activity in several reports, and is presently an integral part of many first line drug combinations in these tumor categories. One may question the dose and schedule of drug administration that was chosen in this study, but in view of the poor response rate it would be difficult to justify alternate treatment schedules in future studies in these tumor types. Furthermore, a prospective randomized trial comparing VM26 and VP16-213 would offer little additional information since the advantage of VP16-213 over VM26 at least in small cell carcinoma of the lung would appear to be obvious.

Other solid tumors are currently being evaluated and will be the subject of another report.

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Letter to the Editor

Adriamycin in Ovarian Cancer Patients Resistant to Cyclophosphamide

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INTRODUCTION

In the management of ovarian cancer patients at stages III and IV a consistent benefit can be obtained by the use of alkylating agents. Melphalan (L-PAM) and Cyclophosphamide (Cy) are widely used because of their relatively low toxicity. About 50% of patients obtain benefit for periods of about 1 yr from these drugs [1, 2]. It is however clear that a second line of therapy must be available for both non responders and relapsing patients.

Despite its structural and pharmacological differences from the family of alkylating agents adriamycin (AMD) was found active on ovarian cancer [3, 4]. These data together with previous conflicting reports of its activity as second line therapy [3, 5] led us to test its activity in a homogeneous population of patients previously treated with a protocol consisting of Cy low doses.

PATIENTS AND CRITERIA FOR THE STUDY

The study comprises 38 patients, median age 53 (27:73), life expectancy more than 3 months, performance status >70, with ovarian cancer stages III, IV previously treated with surgery and cyclophosphamide (Cy) (100 mg/day p.o.); 12 had relapsed after response to Cy and 26 were non-responders; in the latter group 9 were stationary and 17 in progression.

The regimen employed was: ADM 60 mg/m^2 by rapid i.v. injection every 3 weeks. Response was evaluated as ≧50% regressions; progression as ≧50% of measurable masses of new lesions, no change as less than 50% variation in the size of the original lesions. All the patients were evaluated after 3 cycles of ADM.

RESULTS

The table reports the response and survival in groups of patients divided according to their previous response to CY. Out of the 38 patients entering the study only 3 responded to ADM (7.89%, confidence limits for $P=0.05$ 1.66–21.38). The median survival of the 9 patients with a stationary,

Table. Response to ADM

Status after Cy therapy	Second line treatment			
	No.	Responders	No change	Progression
Progression after response	12	2	0	10
Stationary	9	0	9	0
Progression	17	1	0	16
Total	38	3	9	26
Survival (months)		8 (4–11)	11 (6–17)	4 (2–10)

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slow growing tumour was longer (11 months, 4–15) than responding patients (8 months, 6–17) and obviously much higher than patients in progression (4 months 2–10). Five patients are still alive: in one of them, after a partial response it became possible to remove the residual tumour surgically and this patient is alive with no evidence of disease after 15 months. One patient is alive with no change at 4 months and 3 patients are in progression at 3, 3 and 4 months respectively.

The treatment was generally well tolerated and never required admission to hospital. Hematological toxicity was observed in 25% of the women; mean WBC was 3200/mm^3 (1200–6000) mean platelets 130,000 (40,000–420,000), nausea vomiting and alopecia occurred in all cases. No patient suffered cardiac toxicity.

DISCUSSION

Our results indicate that ADM has scant effect in ovarian cancer patients resistant to Cy. This result is not in contrast with the encouraging results obtained with ADM as first line chemotherapy [3, 4, 6], but confirms previous data on its low efficacy as second line therapy [3, 5] in a population resistant to a low dosage of Cy which might be expected to include patients sensitive to more aggressive therapy.

No statistical differences can be found among all the results in the literature and in fact in series of about 40 patients the percentage of response would have to be at least 35–40% to reach a statistically significant level. That ovarian cancer patients resistant to first line chemotherapy do not respond to other treatments has been demonstrated with many drugs such as 5FU [5], high dose MTX [7], high dose Cy [8, 9], *cis*-diamminodichloroplatinum (*cis*-DDP) [10]. It could however be argued that 5FU and MTX have poor activity even in first line therapy and that Cy and *cis*-DDP may develop cross resistance with the alkylating drugs given in first instance. The finding however that ADM behaves in the same manner leads to the hypothesis that ovarian cancer in patients who respond to chemotherapy may have some different features from the ones that show immediate or secondary resistance.

Certainly other causes may contribute to making response improbable such as the size of the tumour, its growth rate, or circulatory efficiency in resistant tumours.

To conclude, it seems that, in the management of ovarian cancer, very little can be gained by sequential treatments, and that better efforts may be expected by improving the first therapeutic attack.

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Letter to the Editor

Rotenone: A Possible Environmental Carcinogen?

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IN A PREVIOUS paper [1] we reported that the respiratory inhibitor rotenone induces a high incidence of mammary fibroadenomas in the albino rat. In a second report [2] we pointed out that rotenone was also inducing the tumors when administered orally. We now report information about the uses of rotenone as an insecticide, pesticide and piscicide which may induce the consideration of this compound as an environmental carcinogen.

Initially [1] we mentioned the use of rotenone as an insecticide in small quantities because the Section of Agricultural Plagues of the Spanish Ministry of Agriculture evaluated the world consumption of rotenone extrapolating for the world population the low Spanish consumption. Further investigations through various state agencies in the United States and an exhaustive bibliographic search in the not readily available literature revealed, however, that:

1. There is a massive consumption of rotenone as an insecticide in superindustrialized countries, especially in the U.S., Great Britain, Sweden, Denmark, Finland, Israel, Canada and Norway. The United States does not produce all rotenone needed for its consumption and must import substantial quantities of rotenone yearly. A minimum of ten million pounds of rotenone and rotenone products (cube and derris powder) were imported in the U.S. in 1950. The importation of rotenone diminished since then, reaching one million pounds in 1972. The total yearly consumption of rotenone in the U.S. presently is estimated at 15 million pounds.
2. Rotenone is used in the U.S. mainly as an agricultural insecticide and as a piscicide. The use as an agricultural insecticide is small, presently in fields (mainly fruit) but, however, is massively used in private household gardens of the American home [3, 4]. As a piscicide, rotenone is used for total killing or for selective killing in lakes, rivers, fisheries, water reservoirs and water plants [5–9]. Through this use, rotenone contaminates drinking water. The concentration of rotenone in drinking water is not available at present but it is enough to cause a characteristic odor and taste [5, 6].
3. Rotenone is also used in the U.S.A. for parasitic controls in cats, dogs and cows but with this use no ingestion of rotenone by humans is foreseen.

Figure 1 depicts the uses of rotenone and its possible ways of arrival to the human female in the U.S.A. The ingestion in consumed fresh or cooked vegetables grown in private household gardens and the ingestion with drinking water are considered the main possible environmental hazards. In Table 1 an experiment is shown in which we have investigated the content of rotenone in green beans cultivated in winter in our laboratory under household garden conditions, using a commercial insecticide containing rotenone. The treated beans contained from 14 to 58 μg of rotenone per gram of beans when ready to be cooked. This amount is well within the order of magnitude of the carcinogenic potency of rotenone (0.1 mg of rotenone daily during 30 days is enough to cause tumours in the rat).

As rotenone is carcinogenic in the rat at very low doses by oral administration and the ingestion of rotenone by humans in substantial amounts, it is more than a possibility, we believe, that rotenone could possibly be considered as an environmental carcinogen. Such a consideration, however, would not guarantee that rotenone is a causative agent of the human mammary tumors in the absence of well founded epidemiological studies.

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Table 1. *Content of rotenone in green beans treated with rotex* 2.5

	Beans	Leaves	Stems	Roots
Control plants	N.D.	N.D.	N.D.	N.D.
Experiment A	58	850	N.D.	N.D.
Experiment B	14	420	N.D.	N.D.

N.D.: Not detectable.
The numbers express micrograms of rotenone per wet weight of plant material. Each experiment is the average of the data obtained from two flowerpots. Rotex 2.5 is a Spanish brand (Grima Laboratories, Valencia, Spain) of insecticide containing 2.5% rotenone. The beans were cultivated in Madrid (Spain) during the winter months, exposed to the sun, air and rain and were collected at a size of 5–10 cm. The plants in experiment A received, in the last two months of growth, three pulverizations with Rotex 2.5, one every 15 days. The plants in experiment B received two pulverizations, one every 30 days. The plants were sprayed according to norms for the use of Rotex 2.5 in agriculture. The determination of the rotenone content was made by gas chromatography as previously described [7] except that we included a previous purification of the chloroform extracts in thin layer chromatography [9].

Although an epidemiological study of rotenone in household gardens and mammary tumor incidence would be highly desirable, this may be perhaps an impossible study since most individuals would not remember what pesticide they used in their garden 10, 20 or 30 yr ago. The purpose of this report is to call to the attention of the scientific community and state organizations with interest in possible environmental hazards to the fact that rotenone is a rat carcinogen by oral route and which is possibly reaching the human female in certain countries in substantial amounts.

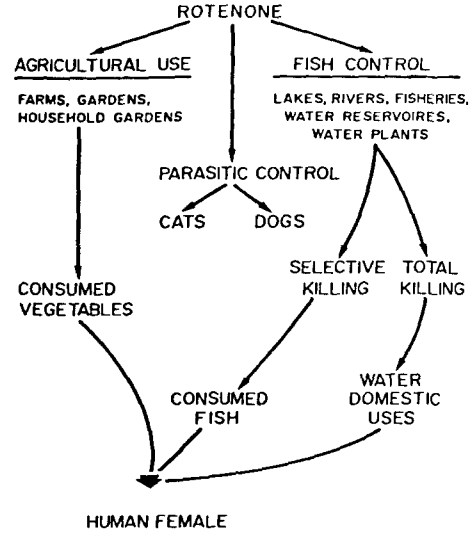

Fig. 1.

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