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Chemotherapy and Surgery of Spontaneous Tumors of Mice

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INTRODUCTION

THE transplantable tumor has been the principal tool in the experimental evaluation of anticancer agents. Primarily this has been justified by convenience and the need for standardized methodologies. Further, some correlation has been noted between the chemotherapeutic responses of human tumors and transplantable mouse tumors, e.g. acute lymphoblastic leukemia of children and the transplantable mouse leukemia L-1210 [1, 2].

Questions may nevertheless be raised as to the appropriateness of the use of transplantable tumors. It has been shown by Woolley *et al.* [3] and Scholler *et al.* [4] that spontaneous tumors are more resistant to chemotherapy than are similar transplanted tumors and that, although transplanted tumors may be affected or eradicated by chemotherapy, the corresponding autochthonous tumor may be unresponsive. In recent studies Chirigos *et al.* [5] and Glynn *et al.* [6] in investigations on the chemotherapy of Moloney virus-induced leukemia also observed a relative resistance of viral-induced autochthonous tumors as compared to corresponding transplantable tumors. Hirschberg, in a comprehensive survey of patterns of response of animal tumors to anticancer agents, concluded that the available information indicates that spontaneous and induced mouse tumors are less readily inhibited by chemotherapeutic agents than are transplanted neoplasms [7].

A number of investigations have indicated that the effectiveness of chemotherapy may decrease as the amount of tumor in the animal is increased. In the experimental chemotherapy of transplanted tumors, Goldin *et al.* [8] have reported on the marked refractoriness to treatment of the advanced tumor albeit they eventually achieved success against advanced mouse leukemia, L-1210, through use of appropriate drugs [9]. Goldin *et al.* [10] and Skipper *et al.* [11] found that the effectiveness of therapy with a folic acid antagonist diminished as the number of cells in a leukemic inoculum was increased. Surgical excision of established transplantable tumors increased the effectiveness of chemotherapy [12–15]. Similarly, surgery and adjuvant chemotherapy was reported to increase the percentage of 'cures' of spontaneous mammary mouse carcinoma, as compared with surgery or therapy alone [16–18]. Whether the refractoriness to treatment of the autochthonous tumor is essentially similar to that of the advanced transplanted tumor is then a possibility.

Our experience in two investigations into the treatment of spontaneous mouse tumors with chemical agents, surgery, or both are presented in this report.

METHODS

Experiment 1

In this experiment, conducted over a period of 7 months, female C_3H/HeN mice bearing spontaneous tumors [19]* were employed. They were obtained from the N.I.H. breeding colonies following detection of the tumors in the course of routine handling.† Age data for the

*Although pathology was not done on the current tissues this strain has been reported to have a high incidence of mammary tumors [19].

†The NIH Animal Production Unit kindly provided tumorous mice from its breeding colony.

mice were not available, though such mice are not ordinarily kept beyond 9 months as breeders.

On receipt, mice varied markedly in their body weight, general physical appearance, and in the size of their tumors; some mice had multiple tumors. According to the order of receipt from the breeding colony, mice were assigned to one of four treatment groups: untreated controls; daily subcutaneous injections of either 50 mg/kg 3'5'dichloromethotrexate (DCM) dissolved in 2% $NaHCO_3$*, or 60 mg/kg 6-Mercaptopurine (6-MP) dissolved in dilute NaOH and neutralized with dilute HCl, or 50 mg/kg Cytoxan dissolved in N-saline. Statistical randomization procedures were employed in determining the order of assignment. Mice were caged individually. Treatment was started on the day of receipt and was continued until eventual death of the mice. Tumors were palpated and mice weighed weekly, though initially more frequent palpation measurements were made.

Experiment 2

This experiment was directed towards evaluating the efficacy of treatment of spontaneous tumors in female C_3H/HeN mice by surgery, or by treatment with Cytoxan, 180 mg/kg injected subcutaneously at weekly intervals, or by combined surgical-chemotherapeutic procedures.

The great variability among mice in the first experiment suggested the need for standardized procedures to achieve uniformity. To this end we maintained in our own laboratory 200 C_3H/HeN female mice, obtained in weekly groups of fifty each from the breeding colony, and initially 4 weeks old. The female mice were caged together with C_3H male mice and permitted to breed; they were not, however, permitted to nurse their off-springs. Mice were examined at weekly intervals for palpable tumors, the first such tumors being observed in the eighth month (thirty-second week) following the average time of birth of the mice. By the twelfth month more than half of the mice (109/200) had developed palpable tumors and had been admitted to the experiment.

The mice found with palpable tumors each week were assigned at random to one of the four treatment groups: untreated controls; surgery only; cytoxan only; surgery followed the next day and at weekly intervals thereafter by cytoxan. Simple surgical removal, by techniques previously described [15], of grossly visible tumor tissue was performed under nembutal anesthesia and the wound closed with clips. Tumorous mice admitted to the experiment were caged individually and observed daily for general appearance, weekly for weight changes, growth or regrowth of old tumors or appearance of new tumors.

*The drugs employed in these experiments were obtained from the Cancer Chemotherapy National Service Center.

RESULTS

Experiment 1

The various panels of Fig. 1 show the significant results, individually for each mouse, of this experiment. The upper control panel shows that untreated mice survived for as little as 5 days or for as much as 117 days (median 40 days) following admission to the experiment. This extreme variation in survival time could be related to the great differences in initial tumor sizes, the diameter of the largest tumor in a mouse varying from 7 to 28 mm* The control data do indicate that with increasing initial tumor size the survival time of mice following admission into the study decreases. But even for a fixed initial tumor size there remains considerable variation in survival times.

The corresponding three drug treatment panels also show considerable variability in survival times and do not indicate any of the three drugs to be efficacious in prolonging the life of tumorous mice (medians: DCM, 36 days; 6-MP, 26 days; Cytoxan, 26 days); if anything, survival time has been clearly reduced for the 6-MP and Cytoxan groups. Suggestive of some possible efficacy for the DCM is the occurrence in that group of two cases of quite prolonged survival time.

The bottom panels of Fig. 1 show the effect of drug treatment on tumor size. To take into account the great variation in initial tumor sizes, the panels show the diameter of the largest tumor 9 or 10 days after beginning of treatment plotted against the initial or day 1 largest single tumor diameter.† The diagonal lines shown on these lower panels help judge changes in tumor size over the interval. Tumor growth is indicated by points falling above the diagonal line, stasis by points falling on or near the line, regression by points falling below the line. Comparing the lower panels, it can be

*Volumewise this could reflect on the order of a 100-fold difference in tumor sizes.

†This day, 9 or 10, was selected for comparison purposes as a compromise. With too early a day there would be inadequate opportunity for treatment to affect tumor size; with too late a day, selection effects, because of death of animals, could have become important.

seen that untreated controls typically showed tumor growth while the treated groups showed lesser amounts of growth and perhaps also some degree of stasis or regression. But such antitumor effects of therapy were not accompanied by increases in survival time, as already noted.

Experiment 2

Figure 2 and Table 1 present the outcome of the second experiment.

For each animal in each experimental group, Fig. 2 shows the survival time following tumor appearance and initiation of treatment. Whether death was with none, one, or multiple tumors is indicated. Where tumors were excised surgically and subsequently recurred, it is indicated by the symbol R.

From Fig. 2 it is possible to gauge the role of animal age at time of tumor appearance, and the control panel data do suggest that tumors in older mice do take longer to kill than do tumors in younger mice. It may be noted from the figure that tumors did not appear until week 32.

Table 1 summarizes the data of Fig. 2, with results combined for all mice in an experimental group irrespective of week of tumor appearance. From the Table the following can be seen:

1. Mice on Cytoxan only had the shortest survival time, median survival times being substantially equal for the three remaining groups. The decrease in survival for the mice treated with Cytoxan alone appears to be attributable to increased drug toxicity in the presence of a large tumor mass, since longer survival times were observed when the same treatment was given to mice which had undergone surgical removal of the tumors. The wide variation in individual survival time for all groups should also be noted.

2. Late deaths, after day 50, without tumor occurred 9 times in the group receiving surgery plus Cytoxan but not at all in the group receiving surgery only. With Cytoxan alone only one mouse died late without tumor.

3. Excised tumors recurred in twenty of twenty-eight mice on surgery only, but in only three of twenty-eight mice which, in addition, received Cytoxan.

4. The occurrence of new tumors was reduced in mice on Cytoxan, whether or not accompanied by surgery.

DISCUSSION

In studies with surgery and adjuvant chemotherapy in mice with spontaneous mammary carcinoma, Martin obtained a higher percentage of 'cures' for surgery plus Cytoxan than for surgery or Cytoxan alone [16, 17]. Also surgery plus combination chemotherapy with thioguanine and mitomycin C or uracil mustard and mitomycin C prevented recurrence of surgically removed spontaneous mouse tumors in approximately 50 per cent of the animals. Surgery plus chemotherapy with only one of the drugs prevented recurrence in 20–30 per cent of the animals [18].

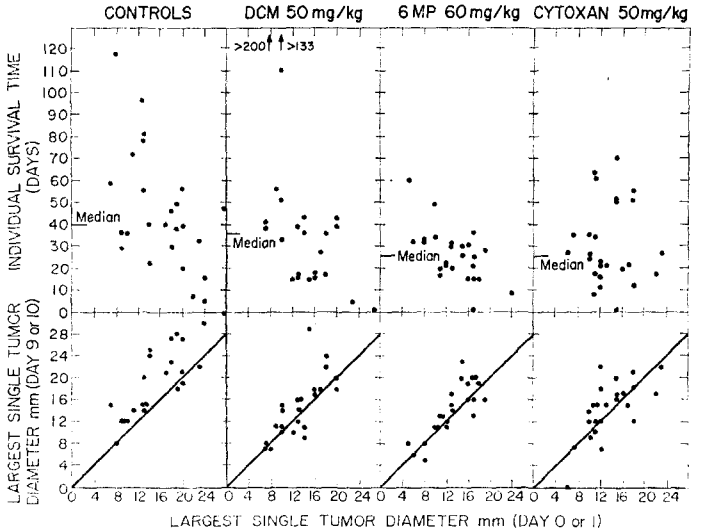

Fig. 1. *Efficacy of various drug treatments for reducing tumor size and prolonging survival time of C_3H/HeN mice bearing spontaneous mammary tumors. The panels show the individual survival times and the maximum single tumor diameter on day* 9 *or* 10 *as a function of the maximum single tumor diameter when the mouse was entered into the study. The diagonal lines in the lower panels indicate where a point would fall if there was no change in tumor diameter over the early treatment period.*

In the current experiments, 3'5'dichloromethotrexate, 6-mercaptopurine and Cytoxan were effective in inhibiting tumor growth. The drugs, however, failed to increase the survival time of the animals.

Although therapy with the combination of Cytoxan and surgical enucleation of tumor did not result in tumor free survivors or in an increase in the survival time of the animals, the evidence does indicate that surgery plus

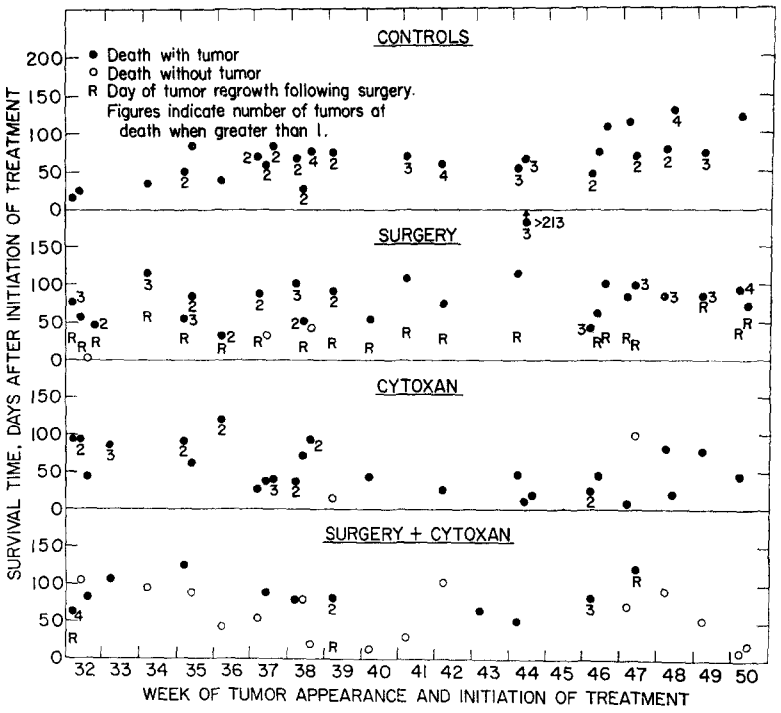

Fig. 2. Efficacy of drug treatment and of surgical removal, separately and in combination, in the therapy of spontaneous tumors arising in a defined cohort of C3H/HeN mice. The figure shows for each mouse when its tumor appeared and its therapy was initiated and how long the mouse subsequently survived. The occurrence of new tumors subsequent to treatment initiation and the time of recurrence of excised tumors are indicated. New tumors have occurred when more than one tumor is indicated at time of death or if any tumors are indicated at death where the excised tumor is not shown to have regrown.

Table 1. Effect of surgery and Cytoxan, alone and in combination, in the treatment of spontaneous tumors in C3H female mice

	Controls	Surgery only	Cytoxan only	Surgery + Cytoxan
No. of mice	26	28	27	28
Survival time, days median	70	81	45	75
range	18–131	0–>213	9–119	1–124
Deaths without tumor after day 50, no.	0	0	1	9
Tumor recurrences, no.		20		3
median day		29		29
range		14–78		15–113
Deaths with multiple tumors, no.		16		3
	17		8	
Mice developing new tumors, no.		16		10
Average no. of new tumors				
all mice	1·0	1·1	0·4	0·5
mice with new tumors only	1·6	2·0	1·2	1·4

Cytoxan was more effective in the control of tumor growth than the surgery employed or chemotherapy alone. With the combination of surgery and weekly administration of Cytoxan there was a marked increase in the incidence of cases in which no tumor growth was evident at the time of death since in these animals the surgically removed tumors failed to regrow and new tumor growth was prevented. Surgery plus chemotherapy also resulted in a decrease in the number of mice that succumbed with multiple tumors.

The limitations to therapy with these drugs and with surgery plus chemotherapy would appear to be the toxicity of the drugs and the refractoriness to treatment of these spontaneous tumors. The continuous treatment in the current experiments increased the risk of deaths attributable to toxicity, and progressive weight loss in many of the mice suggests that this may have been an important contribution to death. Nevertheless, in neither drug-treated group did continuous Cytoxan administration wholly prevent growth of new tumors or regrowth of excised tumor. In fact new tumors appeared in mice where Cytoxan apparently prevented the regrowth of surgically excised tumors.

The current studies lend support to the concept that a decrease in tumor mass may improve the therapeutic effectiveness of drugs [10, 11, 12, 14, 20]. The reduced number of tumor cells may be more vulnerable to adjuvant chemotherapy and if immune mechanisms are implicated they may contribute to enhanced therapeutic effect against a reduced tumor mass [16, 17]. Such attempts to improve therapy of human tumors have been reported by clinical investigators [13, 21–28]. Further investigation of surgery and chemotherapy of spontaneous tumors is undoubtedly warranted.

RESUME

Un traitement quotidien par le 3'5'dichlorométhotrexate, la 6-mercaptopurine ou le cyclophosphamide retarde la croissance de tumeurs mammaires spontanées de souris C3H/HeN. Cependant, sauf de rares exceptions, la survie des souris n'est pas prolongée.

Une exérèse chirurgicale des tumeurs spontanées, associée à un traitement hebdomadaire par le cyclophosphamide, n'augmente pas la survie. Néanmoins, la combinaison semble être plus efficace que la chirurgie seule ou que le cyclophosphamide seul pour contrôler la croissance des tumeurs. Elle augmente l'incidence de cas dans lesquels on ne peut mettre en évidence aucune croissance tumorale — que ce soit sous forme d'une récidive locale après exérèse ou formation d'une nouvelle tumeur — au moment de la mort. De plus, il y a un moins grand nombre de souris qui meurent avec des tumeurs multiples. Cependant, avec l'association de la chirurgie et de la chimiothérapie, de nouvelles tumeurs apparaissent malgré la poursuite du traitement.

Ces données semblent appuyer l'hypothèse que la réduction de la masse tumorale augmente l'efficacité de la chimiothérapie.

SUMMARY

Daily treatment with 3'5'dichloromethotrexate, 6-mercaptopurine or Cytoxan retarded the growth of spontaneous mammary tumors in C3H/HeN mice. However, with possibly a few exceptions, no increases in survival time of the mice resulted.

Surgical excision of the spontaneous tumors plus weekly treatment with Cytoxan did not result in any increases in survival time of the mice. The combination, nevertheless, appeared to be more effective than surgery alone or Cytoxan alone in controlling tumor growth. It increased the incidence of cases in which no tumor growth, whether excised tumor regrowth or new tumor formation, was evident at the time of death. In addition, there was a decrease in the number of mice that died with multiple tumors. However, with the combination of surgery and chemotherapy, new tumors arose despite the continuation of therapy.

The data appear to lend support to the concept that reduction in tumor mass may augment therapeutic response.

ZUSAMMENFASSUNG

Die tägliche Behandlung mit 3'5' Dichloromethortexat, mit 6-Mercaptopurin oder mit Cytoxan verlangsamte das Wachstum von spontanen Brusttumoren in C3H/HeN-Mäusen. Trotzdem wurde, von einigen möglichen Fällen abgesehen, keine Verlängerung der Lebenspanne der Mäuse erzielt.

Die chirurgische Entfernung der Spontantumoren kombiniert mit wöchentlicher Anwendung von Cytoxan führte zu keiner Verlängerung der Lebenspanne der Mäuse. Trotzdem schien diese Behandlungsmethode das Tumorwachstum besser zu kontrollieren als chirurgische Behandlung oder Cytoxananwendung allein. Die Anzahl der Fälle, bei denen nach dem Tode kein Geschwulstgewebe, sei es ein Rezidiv oder ein Neugewächs, zu finden war, schien grösser zu sein. Ausserdem nahm die Anzahl der Fälle, die nach dem Tode multiple Tumoren aufwiesen, ab. Trotzdem entstanden neue Geschwulste, selbst bei ununterbrochener Behandlung, wenn die Kombination von Chirurgie und Chemotherapie angewandt wurde.

Diese Resultate scheinen die Hypothese zu bestätigen, dass die Verminderung der Tumormasse die Wirksamkeit der Chimiotherapie erhöht.

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Effect of 1-adamantanamine Hydrochloride on Rous Sarcoma Virus *in vitro* and *in vivo*[*†]

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INTRODUCTION

ADAMANTANAMINE hydrochloride or amantadine hydrochloride inhibits influenza virus infection in tissue culture, chick, embryos and mice [1]. Rubella virus infection is also inhibited by amantadine hydrochloride in tissue culture [2]. The viruses causing tumours in fowls resemble myxoviruses in some of their characters [3] and it was therefore of interest to test the effect of amantadine hydrochloride on Rous sarcoma virus.

MATERIALS AND METHODS

Rous sarcoma virus (RSV) — The original virus used for these experiments, a Rous no. 1 strain, was kindly supplied by Dr. R. J. C. Harris, Imperial Cancer Research Fund, Mill Hill, England. In the Department of Virology, University of Helsinki, the virus has been passaged in young chickens since 1959. Virus was prepared from chicken tumours according to methods describing in detail in an earlier paper [4].

Cell cultures — Monolayers of chick embryo cells in plastic Petri dishes were prepared from chick embryos 9–10 days of age according to a method described elsewhere [4].

Virus assay — The titer of RSV was determined either by counting the number of pocks formed by RSV on the chorioallantoic membrane (CAM) of fertile chicken eggs as previously described [4], or by counting the number of foci produced by RSV in monolayers of chick embryo cells according to the method of Rubin [5] and described in detail in another paper from this laboratory [6].

Adamantanamine hydrochloride — The synthesis of the compound was performed at the Research Laboratories, Medica Ltd. For the experiments proper the drug was dissolved in phosphate buffered saline, pH 7·6 and used in the concentrations mentioned in the text.

Inhibition experiments were done by adding amantadine hydrochloride to chick embryo cells infected with RSV or by injecting the drug into the yolk sac or onto the CAM of fertile chicken eggs prior to or simultaneously with the infection of the CAM with RSV. The number of foci in the cell monolayers or the number of pocks on the CAM were compared to the number of foci or pocks produced by RSV in untreated monolayers or eggs. Finally the production of tumours by RSV was compared in untreated chicks and in chicks treated with amantadine hydrochloride. White Leghorn chicks from the Saarioinen Farm, Sahalahti, were used.

The details of the experiments are presented in the text.

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†Presented at the Interatinonal Symposium on the Biological Characterisation of Human Tumours, Abbaye de Royaumont, France, 27-28 May 1965.

Table 1. Effect of amantadine hydrochloride (AA-HCl) on focus formation by RSV in chick embryo cell monolayers

Amantadine hydrochloride (μg/ml)	Number of RSV foci		
	AA-HCl at adsorption and in overlay*	AA-HCl in overlay only†	
		Exp. 1	Exp. 2
40	0	2	0
20	0	4	50
10	3	21	115
5	13	17	500
0	26	26	500

*RSV was allowed to adsorb to cells in the presence of AA-HCl. After 60 min unabsorbed virus and AA-HCl was pipetted off and monolayers covered with agar containing AA-HCl.
†RSV was adsorbed as above without AA-HCl.

RESULTS

Amantadine hydrochloride was first tested on RSV in chick embryo cells by using a concentration of 40 μg per ml of cell culture fluid. When the drug was present at the time of virus adsorption a marked decrease in the number of foci produced by RSV in the cell monolayers was seen.

In the next experiment different concentrations of amantadine hydrochloride were tested. In some experiments virus was allowed to adsorb to the cell monolayers in the presence of amantadine hydrochloride and in addition the same amount of the drug was added to the agar overlay. In other experiments amantadine hydrochloride was added to the agar overlay only, after the adsorption of the virus to cells. In both cases the decrease in the number of RSV foci paralleled the amount of amantadine hydrochloride used (Table 1). The greatest inhibition was, however, obtained if the drug was present at the time of virus adsorption.

Concentrations up to 20 μg of amantadine hydrochloride per ml did not cause any visible changes in the cells. With 40 μg of the drug per ml cell culture fluid the cells appeared, however, somewhat impaired. In the following experiments cells transformed by RSV were cultivated in the presence and absence of amantadine hydrochloride. The dose used was 20 μg per ml of culture fluid. The drug did not affect the cells and the yield of RSV was about the same in treated and untreated cultures and rather higher in the treated cultures.

The effect of amantadine hydrochloride was next tested on RSV in chick embryos. In one experiment 500 μg of the drug was inoculated into the yolk sac of 7-day chick embryos. Three days later RSV was inoculated onto the CAM. In the second experiment 250 μg of the drug and RSV were inoculated simultaneously onto the CAM of 10-day chick embryos. Only an insignificant reduction in the number of RSV pocks was seen on the CAM of treated eggs compared to the number of RSV pocks on the CAM of eggs treated with saline instead of amantadine hydrochloride (Table 2).

The following experiments were carried out in chicks about 4 days of age. RSV was injected into the right breast muscle. Two doses of RSV were used. Amantadine hydrochloride was given daily subcutaneously and/or intramuscularly in the leg. Control groups received physiological saline instead of amantadine hydrochloride. One group received amantadine hydrochloride only and served as a control for the toxicity of the drug. One group, finally, received simultaneously amantadine hydrochloride and RSV in the breast muscle and thereafter daily injections of the drug as above (for details see Table 3).

The initial dose of 5 mg amantadine hydrochloride twice daily corresponding to about 100 mg/kg bodyweight turned out to be toxic. The chicks got diarrhoea and became cachetic.

Table 2. Effect of amantadine hydrochloride (AA-HCl) on the formation of pocks on the chorioallantoic membrane (CAM) by Rous sarcoma virus

Dose of amantadine hydrochloride	Mean number of pocks on the CAM	
	Control*	Test
500 μg yolk sac	65	54
250 μg CAM	35	26

*Embryos pretreated with physiological saline solution instead of AA-HCl.

Table 3. *Effect of amantadine hydrochloride (AA-HCl) on RSV induced tumours in chicks*

Treatment	Dose of RSV FFU/0·2 ml*	Number of chicks	Day of appearance of tumours†	Mean survival day‡
Saline§	5×10^3	10	8	16·0
AA-HCl‖	5×10^3	9	8	13·6
AA-HCl¶	5×10^3	8	12–13	17·9
Saline§	5×10^2	9	9–10	17·7
AA-HCl‖	5×10^2	7	10–11	18·9

*Inoculated into right breast muscle.
†Tumours palpable in at least 50 per cent of chicks.
‡$\frac{\Sigma \text{ (No. of chicks alive each day to day 21)}}{\text{Total No. of chicks in the group}}$
§0·2 ml twice daily subcutaneously and/or intramuscularly in the leg.
‖Administered as above. First day: 5 mg × 2; second day: 2·5 × 2; third day: 2·5 mg × 1; fourth day: 2·5 mg × 2; fifth day: no treatment; from eight day onwards: 2·5 mg × 2.
¶RSV inoculated with 5 mg AA-HCl treatment continued with AA-HCl as above.

In addition, convulsions were seen immediately after the injections. The dose was therefore reduced to 2·5 mg twice daily. In spite of this some chicks died and treatment was omitted for a few days. Thereafter treatment was, however, continued with 2·5 mg twice daily. This dose was well tolerated and after the initial decrease in weight chicks obtaining amantadine hydrochloride increased in weight as well as chicks obtaining saline (Fig. 1). However, at the end of the experiment the daily dose was thus only about 25 mg/kg bodyweight.

With the higher dose of virus there was no effect of amantadine hydrochloride (Table 3). Treated chicks died rather earlier than chicks receiving saline only. With the smaller dose of virus a slight delay in tumour appearance was observed and the mean survival time was about one day longer. Only in the group receiving amantadine hydrochloride together with RSV in the breast muscle was there a clear delay in tumour appearance but in spite of this the mean survival time was only about two days longer.

DISCUSSION

According to the preliminary experiments presented above the effect of amantadine hydrochloride on RSV in cell cultures was com-

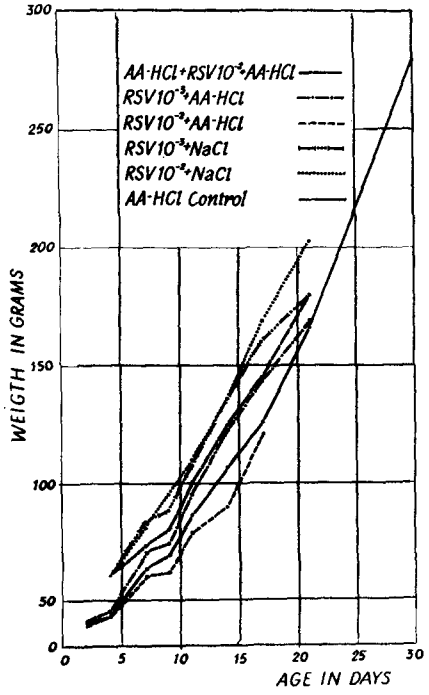

Fig. 1. *Effect of amantadine hydrochloride (AA-HCl) on the weight of noninfected and RSV infected chicks.*

parable to that obtained with influenza virus under similar conditions [1]. As in the case of influenza virus amantadine hydrochloride most probably inhibits the penetration of RSV.

However, when tested *in vivo*, practically no effect was seen on the CAM of fertile chicken eggs and in chicks a delay in tumour appearance was seen only if amantadine hydrochloride was administered simultaneously with RSV into the breast muscle. Even then, however, tumours occurred in spite of continuous injections of amantadine hydrochloride in the leg.

If, as suggested by the experiments in cell cultures amantadine hydrochloride inhibits penetration of RSV, the weak effect *in vivo* may depend on the fact that high enough concentrations of the drug were not obtained at the site of virus production. Another possibility would be that tumour growth to a great extent results from proliferation of transformed cells in which case amantadine hydrochloride would have no inhibitory effect.

Experiments designed to elucidate this question as well as problems concerned with the effect of amantadine hydrochloride on the interaction between RSV and cells are in progress.

Acknowledgement—The excellent technical assistance of Mrs. Annikki Kallio is gratefully acknowledged.

SUMMARY

Adamantanamine hydrochloride caused a dose-related inhibition of Rous sarcoma virus infection in chick embryo cell cultures. However, with the doses used, practically no inhibition could be demonstrated on the chorioallantoic membrane of fertile chicken eggs and, in chicks, a delay in tumour appearance was observed only if adamantanamine hydrochloride was injected simultaneously with the Rous sarcoma virus into the breast muscle. The results and their possible implications are briefly discussed.

RESUME

Le chlorhydrate d'adamantanamine est capable de provoquer une inhibition — liée à la dose — de l'infection par le virus du sarcome de Rous, dans les cultures de cellules embryonnaires du poulet. Néanmoins, avec les doses utilisées en pratique, aucune inhibition ne peut être mise en évidence, sur la membrane chorio-allantoïdienne d'oeufs fertilisés. Un délai dans l'apparition de la tumeur n'aété observé que si le chlorhydrate d'adamantanamine était injecté en même temps que le virus dans le muscle pectoral. Ces résultats et les conclusions que l'on peut en tirer sont brièvement discutés.

ZUSSAMMENFASSUNG

Adamantanamin-hydrochlorid erzielt eine dosisgebundene Inhibition der Infektion von Hühnerembryo-Zellkulturen mit Rous-Sarkomvirus. Die angewandten Dosierungen führten zu keiner Inhibition auf den Membranen von befruchteten Hühnereiern und bei Küken kam es nur zu einer Verzögerung des Auftretens der Tumoren, wenn das Adamantanamin-hydrochlorid zur selben Zeit wie das Rous-Sarkomvirus in den Brustmuskel injiziert wurde.

Die Autoren besprechen kurz ihre Resultate und deren mögliche Auswirkungen.

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Osteopetrosis Virus Growth in the Kidney of Infected Chickens

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INTRODUCTION

THE VIRUS responsible for osteopetrosis in chickens is morphologically similar to the chicken leucosis-sarcoma type, and has been reported to extensively infect tissues of birds suffering from this condition [1, 2]. The progressive infection of the various tissues involved has been described, and it appears that the kidney is the first organ to be infected [2]. As a result of this unexpected observation, several lines of investigation were undertaken to ascertain the nature of this viral infection of the kidney.

There were:

a. To determine if virus isolated from the infected kidney tissue and passaged as cell-free suspensions did in fact have the same pattern of infection as routinely-passaged blood from birds known to be infected with osteopetrosis.

b. To determine if urine obtained from birds suffering from osteopetrosis was itself infective.

c. To carry out a detailed examination of the kidney with the electron microscope in order to investigate the exact distribution of the virus and the changes occurring in the kidney during the course of infection.

As previously shown [2], the virus strain used in these experiments is probably uncontaminated with leucosis viruses, as shown by the uniformity of response when it is injected into day-old chickens.

MATERIAL AND METHODS

Routine passaging — All experiments were carried out using the isolated flock of Brown Leghorn birds maintained at the Poultry Research Centre in Edinburgh. These birds are considered virus-free [3, 4], and no demonstrable tumour viruses have been found since the flock originated. The original osteopetrosis passage material was a sample of infective blood sent by Dr. Holmes from his strain of osteopetrosis, isolated at the Veterinary School of Bristol University. The routine passage of the disease was by intramuscular injection of 0·2 ml of whole blood into day-old chicks.

Virus development in the kidney — 100 birds were injected with blood from birds showing clinical symptoms of osteopetrosis and 2 or 3 were killed at periods of 2–3 days over a period of 12 weeks. Controls consisted of 50 birds injected with 0·9% saline. Small pieces of kidney were fixed and examined using the electron microscope.

Electron microscopy — Immediately after the death of the birds, tissues were fixed in 5% glutaraldehyde and post-fixed in osmium tetroxide [3]. Dehydration and embedding in Araldite were by the usual methods. Specimens were sectioned with a Cambridge microtome, examined with a Phillips E.M. 100 electron microscope and photomicrographs of the specimens were taken using Ilford 5B11 35 mm film.

Passage of virus from the kidneys of birds infected with osteopetrosis — Kidneys from birds with advanced osteopetrotic lesions were dissected out and cell-free preparations prepared by macerating the tissue in distilled water (approx 1/1, W/V) with a high speed mechanical cutter. The cellular debris was removed by centrifugation at 1500 rev/min and 0·2 ml/aliquots of this suspension were injected into day-old chicks. Control experiments were carried out using uninfected kidneys as a source of material.

Infectivity of urine from osteopetrotic birds — Urine was obtained from birds with advanced osteopetrotic lesions. These birds were first tied

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securely to a wooden frame, and cotton wool was inserted through the vent into the cloaca so that the urodaeum was free from faeces. A small plastic funnel was then inserted into the cloaca and held in place by means of strips of elastoplast. Birds treated in this way were left covered with a cloth, in order to keep them quiet, and urine, uncontaminated with faeces, was collected in test tubes. Day-old chicks were given 0·5 ml/doses of urine orally or 0·2 ml/aliquots intra-muscularly. Control experiments were carried out using uninfected birds as sources of urine.

RESULTS

a. Birds injected with kidney extracts. A survey of the distribution of virus particles, and the lesions resulting from the injection of these preparations, is summarised in Table 1. The pattern of infection seems to be very like that found in birds injected with osteopetrotic blood: a low incidence of bone lesions but a high incidence of kidney infection. The kidney extracts used must certainly have contained blood-borne virus, but a high percentage of 'kidney virus' must also have been present. Kidneys of birds infected with these preparations did not show unusually high concentration of virus particles, compared with those of osteopetrotic birds from routine passages, and neither did they show a higher incidence of kidney tumours.

b. Birds infected with urine. Table 2 summarises the effect of the inoculation of infected urine into day-old chicks. Oral administration results in a low incidence of viral infection, and only one in twenty birds developed osteopetrosis, although four showed replication of viruses in their kidneys. However, six out of twelve day-old chicks which survived intra-muscular inoculation showed virus replication in the kidney and three also had periosteal lesions.

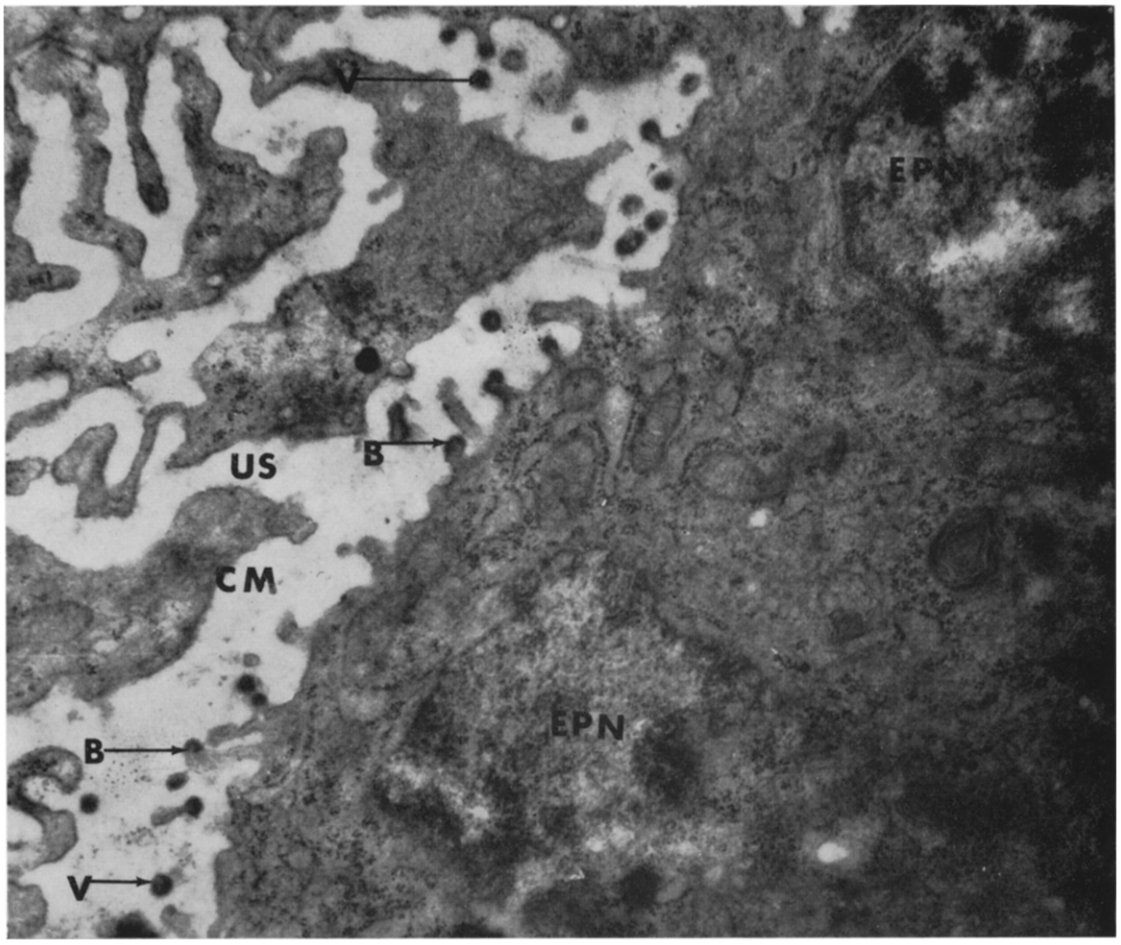
The course of infection in the kidney — The general distribution of osteopetrosis virus particles in the chicken has been reported [2]. Virus particles were first found in the kidney after about 3 weeks. At this time they were very few in number and only in the proximal regions of the nephron and in the glomerulus. There was a progressive build-up of virus in the region of the epithelial cells of the tuft, and at about four weeks frequent budding was seen from the cell membranes of these cells (Fig. 1). This gradual increase in virus production by the epithelial cells appeared to reach a maximum at about 4–5 weeks, and after this time no further increase in concentration could be detected. During this period (age 3–4 weeks) the intra-tubular urine was becoming more and more heavily contaminated with virus particles (Fig. 2). The cells lining the tubule were found to be intimately associated with viruses after the fourth week. Different regions of the tubules could be distinguished easily by the morphology of the cells lining these particular regions [6]. Consequently, it was relatively easy to follow the progressive infection of cells down the tubule. After the fourth week, virus particles could be seen between adjacent cells of the proximal region of the tubules, and at the fifth week, distal tubule cells showed virus particles between their interfaces (Fig. 3). The collecting tubule cells did not appear to be infected to the same extent as the proximal and distal regions, and only very occasionally were virus particles seen between cells of the collecting tubules. The infection of the distal

Table 1. Distribution of osteopetrosis viruses in birds inoculated with cell-free extracts of kidneys from birds with osteopetrosis.

	Extracts from infected kidneys	Extracts from normal kidneys
Number of birds inoculated	20	8
Birds with virus in:		
Kidney	16	0
Periosteum	4	0
Kidney abnormalities:		
Cysts	6	0
Tumours	0	0
Cilia	3	0

All birds were killed at about 12 weeks.

Although Table 1 suggests tumours were not found in the kidneys of birds infected with osteopetrosis virus, other unpublished experiments have occasionally shown kidney tumours containing osteopetrosis-like virus particles in birds infected in a similar way to those described in Table 1.



Abbreviation

B — virus bud; BM — basement membrane; C — blood capillary; CM — cell membrane; END — endothelial cell; EP — epithelial cell; EPN — epithelial cell nucleus; F — fenestrations of glomerular epithelial cell layer; IS — intercellular space; M — mitochondria; US — urinary space; V — virus.

Fig. 1. *Virus budding off from the cell membrane of an epithelial cell in the glomerulus of a kidney from a bird injected with an osteopetrosis virus preparation at* 1 *day old. The age of this bird when killed was about* 5 *weeks.*

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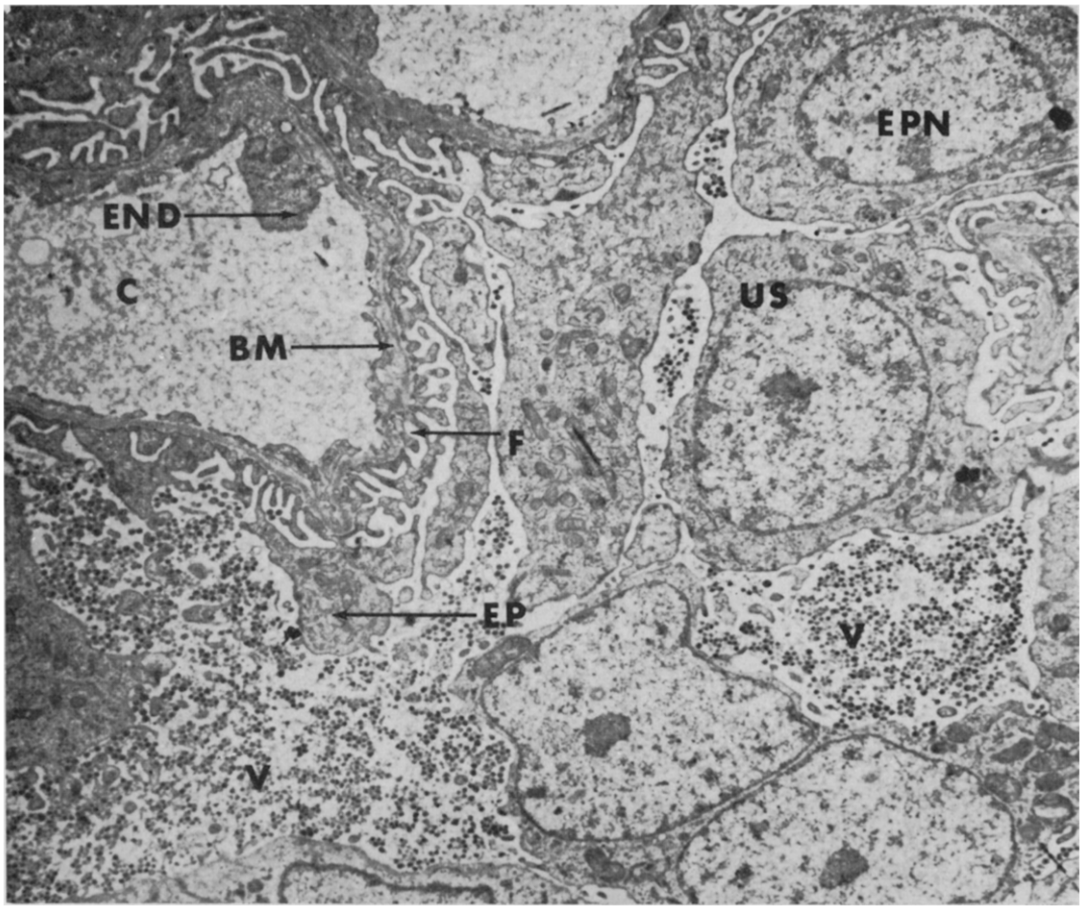

Fig. 2. A relatively low-power micrograph of the glomerular region in a kidney from a bird aged about 12 weeks suffering from osteopetrosis. Large numbers of virus particles can be seen, in the urinary spaces, which will eventually be excreted.

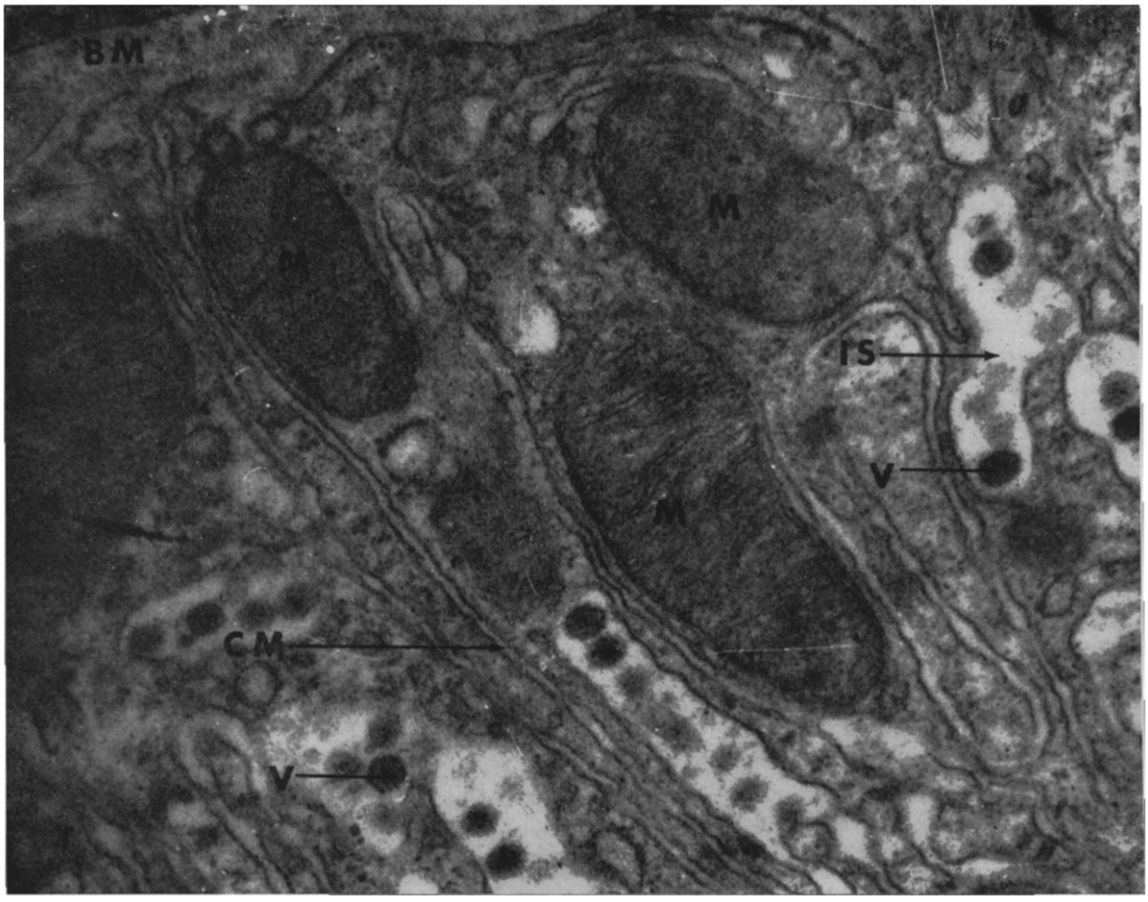

Fig. 3. This micrograph shows virus particles in the intracellular spaces between cells of the proximal convolted tubule. The extensive involutions of the cell membrane can be seen with the virus particles in small "cyst-like" enlargements of the intercellular spaces. No intracellular virus can be detected.

Table 2. Distribution of osteopetrosis viruses in birds inoculated with urine from birds with osteopetrosis.

	Oral inoculation	Intramuscular inoculation	Intramuscular inoculation of normal urine
Number of birds inoculated	20	19	9
Birds with virus in:			
Kidney	4	6	0
Periosteum	1	3	0
Number of birds dead of unknown cause after one day	2	8	4

All birds were killed between 12 and 16 weeks.

and proximal region eventually led to large concentrations of virus in the intercellular spaces between the cell membranes of the tubule cells and the adjacent basement membranes surrounding the tubule. At about 5–6 weeks these concentrations were quite large, and at 6 weeks virus particles were frequently seen in the blood in regions immediately adjacent to these large concentrations of virus. Virus particles were occasionally seen apparently within the basement membranes adjacent to distal and proximal cells (after 5 or 6 weeks). Pinocytosis was observed at the luminal surface of kidney cells in the distal and convoluted regions. Although virus particles were never seen being absorbed by cells, it is probable that this is the route by which these viruses enter and infect kidney tubule cells.

Tubular hypertrophy and cysts, which were occasionally lined with cilia, were infrequently observed, but these conditions are more often found in older birds, 6 months to a year after initial infection with osteopetrosis. [1].

DISCUSSION

Day-old birds, infected with extracts from osteopetrosis-infected kidney tissue, developed osteopetrotic symptoms in a way similar to those infected with whole blood. This does not necessarily mean that the virus replicating in the kidney was capable of producing osteopetrosis, since this tissue contains a lot of blood, and blood itself is a good source of infective virus. Consequently the kidney extracts would contain blood-borne viruses. However, no obvious increase in the degree of kidney involvement was noticed in birds infected in this way. This suggests that the high titre of the virus produced from the kidney cells, found in the cell-free extracts of infected kidneys, merely produced the normal osteopetrotic condition. This supplied further evidence that the virus growing in the kidney was the same as that responsible for osteopetrosis.

Both oral administration and intra-muscular injection of day-old chicks with urine from osteopetrotic birds resulted in virus infections. This also indicates that the virus particles in the urine were infective osteopetrotic particles, and not merely viruses with morphological characteristics similar to those of the osteopetrosis particles. As might be expected, infection by the oral route proved less successful than the intra-muscular route. However, the fact that the urine would infect chickens by these routes suggests that natural transmission may occur through contamination of food or drinking water. Infective urine and infective reproductive organs [7, 8] are probably responsible, respectively, for horizontal and vertical transmission of this disease in the field.

The course of infection in the kidney — Since the object of this work has been to follow the course of infection of the osteopetrosis virus, ciliogenesis has not been considered in detail. However, Sorokin [9] gives an excellent general review of the subject and Campbell, Young and Carr [1] describe the occurrence of cilia in osteopetrotic infected kidneys.

The kidney is probably infected through the blood. Experimental infection results from virus particles entering the blood after inoculation. However, in the field the virus probably enters the blood after absorption by the cells of the alimentary tract. These particles will then be carried to the glomerulus where, probably, they are actively passed from the blood capillary through the basement membrane to the urinary space. Rhodin [6, 10], in general reviews of the kidney structure, and Pak Poy and Robertson [11], in an examination of the avian renal glomerulus, have shown that only the basement membrane separates the blood and the urine at this point. The endothelial cells of the blood capillaries have many small fenestrations, so that these cells do not form a continuous layer over the lining of the capillary. Similarly, the epithelial cells have long slender cytoplasmic processes which extend over the

capillaries. Several authors have shown that particles between 50Å and 200–400Å in diameter, e.g. ferritin, thorotrast, colloidal gold and dextrans, are rapidly passed through the basement membrane of the glomerulus, after being injected into the blood, and accumulate in the urinary space [12–15]. Eventually these particles, which are absolutely biologically inert, pass into the lumen of the kidney tubule, but may be occasionally phagocytosed by the epithelial cells in the glomerular region of the kidney cells lining the tubule.

The passage of virus particles through the basement membrane may be active or passive: this question is open. However, virus particles have been seen at this point, actually in the middle of the basement membrane. As a result of the experiments carried out by Farquhar and Palade [14], and the knowledge that viruses appear to accumulate in the urinary space of the glomerulus, it was concluded that the passage of viruses across the basement membrane is in one direction only and is from the blood to the urine. This conclusion receives further support from the facts that the kidney actively "filters" blood and that viruses have never been seen in the blood in this region. The virus then infects the visceral epithelial cells of the glomerular tuft, probably by a process similar to the absorption of inert particles, as described by Farquhar and Palade [14]. Budding in great profusion takes place here. Consequently, a large amount of virus is produced in Bowman's capsule, is liberated into the urinary space, and then transported in the urine down the lumen of the tubules.

Infection of the distal and proximal tubule cells is probably by a process of pinocytosis. The infected tubule cells then "bud off" virus particles into the spaces between adjacent cells, and between the cells and the basement membrane surrounding the tubule. (Virus which is not shed into the inter-cellular spaces is probably unable to pass into the lumen, since the desmosomes form an impenetrable barrier to their passage.) The virus particles, which accumulate between distal and proximal cells, are then able to pass through the basement membranes surrounding the tubules and move into the blood. The process is assumed to be "one way," viz. the reverse direction to that found in the glomerular region, for two reasons:

1. Active "diffusion" of material back into the blood takes place at this point.
2. Virus particles are observed in small groups in the blood in the capillary network surrounding the nephron, and this is never seen in the glomerular regions.

It is interesting to note that many viruses can most easily be cultured in tissue cultures of kidney cells. *In vivo* studies have also revealed that the viruses responsible for polio [16], myeloid leucosis — B.A.I. strain [17], erytholeucosis — E.S.4 strain [18], and infectious bronchitis [19] all have an affinity for kidney tissues. Furthermore, the susceptibility of the kidney to tumorigenesis is a general characteristic of all animals and not restricted to chickens [20–22]. If all kidneys are susceptible to viral infection of this type, this may be another piece of indirect evidence supporting the hypothesis that many viruses, normally associated with non-cancerous conditions, may occasionally, under certain conditions, be oncogenic.

SUMMARY

An examination of kidneys of chickens aged between 2 days and 12 weeks old, previously infected at 1 day old with osteopetrosis virus preparations, has been carried out using the electron microscope. The progressive infection of the kidney with virus has been followed. Replication of new virus can first be detected in the glomerulus of 3-week-old chickens but gradually cells further and further down the nephron became infected. Older birds have considerable numbers of virus particles in the intercullular spaces in the proximal and distal regions of the nephron and also in the lumen. Virus extracted from the kidney and passaged into day-old chicks appeared to produce a normal osteopetrotic virus infection, suggesting that the virus replicating in the kidney was in fact osteopetrosis virus. Similarly, urine obtained from birds with advanced osteopetrosis lesions and injected orally and intramuscularly into day old chicks also produced osteopetrosis, suggesting a possible route for natural contamination.

RESUME

Les reins de poussins injectés le jour de l'éclosion avec le virus de l'ostéopétrose ont été examinés au microscope électronique, à partir du deuxième jour jusqu'à la douzième semaine.

La replication virale commence dans le glomérule autour de 3 semaines, et l'infection se propage de cellule en cellule le long du néphron. Beaucoup de particules virales se trouvent dans les espaces intracellulaires du néphron et aussi dans la lumière du tube rénal Des extraits acellulaires de ce virus rénal reproduirent le tableau habituel de l'ostéopétrose. L'urine des animaux avec ostéopétrose floride peut provoquer la maladie chez des poussins nouveau-nés, par injection intramusculaire ou administration orale, ce qui peut indiquer le mode de la transmission normale.

ZUSAMMENFASSUNG

Von Kücken die am ersten Lebenstag mit Osteopetrosevirus infiziert norden waren, wurden, als sie ein Alter Zwischen 2 Tagen und 12 Wochen erreicht hatten, die Nieren elektronenmikriskopisch untersucht. So war es möglich die fortschreitende Virusinfektion der Niere zu verfolgen. Virusvermehrung wurde bei 3-Wochen alten Kücken nachgewisen und zwar zuerst im Glomerulus und dann nach und nach immer weiter entlang dem Nephron. Bei älteren Vögeln sah man beträchtliche Virusansammlungen nicht nur in den intrazellulären Räumen der proximalen und distalen Teile des Nephrons sondern auch in den Lumina der Tubuli. Wenn Einstagskücken mit Virus infiziert wurden, welches von solchen Nieren gewonnen war, so entwickelte sich eine typische Osteopetroseinfektion, die daher die Annahme gestattet, dass das in den Nieren sich verbreitende Virus tatsächlich dasselbe ist, welches die Osteopetrose verursacht. Wenn ausserdem Urin von Vögeln mit fortgeschrittener klinischer Osteopetrose an Eintagskücken oral oder intramuskulär verabreicht wird, so resultiert abermals eine typische Osteopetrose. Diese Befunde lassen an die Möglichkeit denken, einen natürlichen Infektionsweg gefunden zu haben.

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Synthesis of Plasma Proteins by the Perfused Rat Liver

Effects of Protein Free Diet, 3′–MDAB and Dimethylnitrosamine

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A GREAT deal of information is now available concerning the mechanism of protein synthesis and the role of DNA and RNA in this process. Most of this information has been obtained with sub-cellular systems only some of which have been derived from mammalian cells. While almost all the available data are in concordance with the widely accepted theory of protein synthesis which has grown up from the original proposal of Watson and Crick [1], certain aspects of the problem are still in dispute [2]. Further work with systems of the types just mentioned should ultimately define with certainty all the chemical reactions necessary for the synthesis of a given protein molecule. Yet while such information should indicate some of the ways in which rates of synthesis may be controlled it seems probable that experiments with intact organs will be necessary to reveal the factors which are actually operative in each particular case *in vivo*.

For studies of the kind the liver has many advantages because by perfusion with heparinised blood it can be maintained in a relatively normal state in which synthesis of most of the plasma proteins and all the proteins which make up the liver cells is continued. In the present work the incorporation of ^{14}C leucine into three of the plasma proteins has been measured. These proteins, fibrin, albumin, and transferrin 1, were chosen because they were known to be synthesised by the liver and because of the existence of convenient methods for isolation.

As yet few investigations relating directly to factors which may alter the rates of synthesis by the liver of the plasma proteins have appeared. However, Burke and Miller [3, 4] measured incorporation of ^{14}C labelled amino acids into the total plasma proteins by perfusion of the livers of rats which had been fed with 3′–MDAB. Whether this substance is able *in vivo* to bring about methylation of nucleic acids with consequent effects on protein synthesis is as yet unclear. On the other hand Magee and Farber [5] have been able to demonstrate that when dimethylnitrosamine is present in the diet both DNA and RNA in the liver are methylated with the formation of 7 methylguanine. In the present experiments livers from rats which had received one or the other of these carcinogens have been perfused. In addition because the relative rates of synthesis of the plasma proteins are known to differ in animals on diets deficient in protein [6], this condition was also investigated. A preliminary report of this work has been given [7].

METHODS

Strain of rats used as liver donors and diets — The rats were of the hooded strain maintained at the

National Institute for Medical Research. Three special diets were used the first which contained 0·06% of 4–dimethylamino–3′–methylazobenzene (to be referred to as 3′–MDAB), for which thanks are due to Dr. P. N. Campbell, was similar to that of Kline, Miller, Rusch, and Baumann [8] and was prepared exactly as described by Campbell [9] and fed *ad libitum*. The rat used as liver donor for perfusion No. 87 was injected intraperitoneally with 40 mg 3′–MDAB partly dissolved in 2 ml olive oil. The perfusion was carried out 49 hr later. The second diet containing 0·01% of dimethylnitrosamine (to be referred to as DMN), for which thanks are due to Dr. P. N. Magee, was M.R.C. diet 41 B to which DMN in arachis oil had been added and mixed as described by Magee and Barnes [10]. This was also fed *ad libitum*. The rat used as liver donor for perfusion No. 98 was injected intraperitoneally with 1·0 ml 1·5% DMN in 0·9% NaCl solution two days before the liver was perfused. The protein free diet was the same as that used by Freeman and Gordon [11], except that for perfusion Nos. 02, 05 and 08, a few drops of a solution of vitamins A, D, K and E, in arachis oil were fed to the individual rats. This diet was kindly supplied by Miss D. P. Woodnott.

Perfusion apparatus and technique — The apparatus and surgical technique were the same as described by Cohen and Gordon [12] 65–75 ml fresh rat blood containing approximately 20 mg (2000 IU) heparin were used for each perfusion. When livers from rats which had been fed on the 3′–MDAB or the DMN diets (see above) were perfused, the first 5 ml blood emerging after cannulation were rejected. 5 μc ^{14}C leucine were added to the blood approximately 20 min after the liver had been transferred to the perfusion circuit. 5–11 hr later the liver was removed, perfused with 20 ml 0·9% NaCl and weighed. Samples of each of the isolated proteins were combusted to CO_2 which was then estimated for ^{14}C in a Packard Tricarb scintillator [13].

Isolation of fibrin — 12 ml of the plasma obtained from each liver perfusion was dialysed for 40 hr against a 13 mM sodium citrate, 2 mM sodium phosphate buffer containing leucine at pH 7·6. After the first 20 hr the buffer was changed. This and all subsequent dialyses were done at 2°. Next the plasma was centrifuged and divided for isolation of fibrin and albumin. 10 ml were used for the isolation of fibrin. This was done by addition of 2 vol 0·9% NaCl 1·1 vol saturated ammonium sulphate as described by McFarlane [14]. Carrier leucine was also added. After standing for several hours the precipitate was removed by centrifuging for 20 min and stirred with 2 ml of the citrate phosphate buffer adjusted to pH 7·0. The solution thus obtained was then dialysed for 20 hr. After removal from the sack and centrifuging, 6 drops of 0·5% protamine sulphate and 4 drops thrombin calcium chloride solution (0·1 mM calcium chloride and 1·0 iu of thrombin) were added and the solution was allowed to clot by standing at 37° for 30 min. After allowing time for the clot to retract it was rolled up and left overnight in 0·9% NaCl. A similar period in water then followed and finally washing with ether: ethanol 1: 3 by vol, drying and weighing.

Isolation of albumin — 2 ml of the plasma after it had been dialysed for 40 hr and centrifuged as described above, was treated with 10 vols 96% ethanol–1% trichloroacetic acid [15, 21] and the precipitate was removed by centrifuging. The supernatant was concentrated by pressure dialysis in the presence of carrier leucine. After concentration, dialysis was continued for a further 72 hr against water which was renewed each day. Finally the solution was centrifuged and the supernatant was frozen dried.

Isolation of transferrin 1—12 ml of the plasma obtained from the liver perfusion was dialysed for at least 20 hr against veronal buffer containing bicarbonate and Fe^{3+} ions at pH 8·6 as described by Gordon and Louis [16]. After this, transferrin 1 was isolated exactly as described by Gordon and Louis [16] except that carrier leucine was present during the first two dialysis stages. The homogeneity of the transferrin 1 after chromatography on DEAE-Sephadex was checked by chromatography of a pooled sample on carboxymethyl-Sephadex C50. This exchanger after equilibration with 5 mM sodium phosphate buffer at pH 6·1 was made into a column 28 × 0·9 cm. After dialysis against the same buffer a solution containing 15 mg of transferrin 1 was applied and eluted at room temperature by means of a gradient produced with 100 mM sodium phosphate at pH 7·4 and a 500 ml mixing chamber. The flow rate was 20 ml per hr.

Radioactivity of liver proteins — At the end of the perfusion 20 ml 0·9% sodium chloride was passed through the liver. A small part of the organ was then homogenised and diluted with 2 vol of 0·9% sodium chloride. The suspension thus obtained was dialysed for 5 days against water containing leucine and sufficient ammonia to maintain the pH between 7–8.

Specific activity of free ^{14}C leucine in plasma and liver during perfusion — Liver and plasma pro-

Table 1. *Specific activity of liver and plasma free ^{14}C leucine during perfusion.*

		Free leucine		Free ^{14}C leucine				
	Time perfused (min)	Present in liver or perfusion plasma as % of amount in normal liver at 20 min		Amount present as % of initial dose†		Specific activity		
						As % of that for normal liver at 20 min		Ratio
Diet of liver donors		Normal	Protein free	Normal	Protein free	Normal	Protein free	Protein free / normal
In liver	20	100*	37	8·8	6·9	100	208	2·08
	36	98	41	7·3	3·1	85	86	1·0
In perfusion plasma	20	306	144	52	46	194	366	1·88
	36	445	136	50	27	126	224	1·78

*Liver wet weight 7·7 g contained 3·93 m mol leucine.
†Initial dose was 1·5 μc ^{14}C l-leucine.

teins were removed by the picric acid method of Stein and Moore [17]. After chromatography on a short column of Dowex 1 to remove picric acid, the eluates were estimated for leucine by means of an amino acid analyser. Aliquots of the solutions after removal of picric acid were concentrated by freeze drying and transferred quantitatively for estimation of total ^{14}C radioactivity. The ^{14}C leucine specific activity values given in Table 1 are based on the assumption that all the radioactivity in the deproteinised solutions was present as free ^{14}C leucine.

Electrophoresis in starch gel — This was carried out exactly as described by Gordon and Louis [16].

Estimation of fibrinogen — This was done by the method of Ellis and Stransky [18] on blood containing 0·1 vol of 3·8% sodium citrate obtained from groups of male and female rats similar to those used as blood donors.

RESULTS

Bases for expressing results and corrections applied — Reid [19] has discussed the various bases on which biochemical data obtained from rats treated with hepatocarcinogens may be expressed. Since expression of the results both per rat and per g liver each has certain advantages, the present data for the specific activities

Table 2. *^{14}C Specific activities of isolated proteins labelled by livers from normal rats and from those on a protein free diet*
Corrections for pool sizes are as in Table 3

Perfusion No.		μc/gC			Perfusion No.		μc/gC		
		Fibrin	Tr 1†	Albumin			Fibrin	Tr 1†	Albumin
75	Normals	0·941	0·720	1·25	83	Protein free diet*	2·39	0·848	0·953
78		1·45	0·544	1·15	58		1·94	0·872	0·945
86		1·22	0·668	1·58	02		2·27	0·980	0·868
09		1·90	0·613	1·21	05		1·54	0·884	1·63
06		1·56	—	1·57	08		2·22	0·824	1·34
07		1·52	—	—					
Average sp act. uncorrected for liver weight μc/gC		1·43	0·645	1·35			2·07	0·878	1·15
SEM %±		9·36	5·97	6·84			7·35	3·08	12·8
Average sp act. corrected for liver weight μc/gC/g liver wet weight		0·164	0·0736	0·157			0·364	0·151	0·202
SEM %±		7·25	11·3	8·90			17·8	13·4	21·0

*7–10 weeks on diet.
†Transferrin 1

Table 3. ^{14}C specific activities of plasma proteins isolated at end of liver perfusion

Perfusion No.		Liver donor % of initial weight	Liver % of normal weight	Sp. act. of isolated proteins μc/gC			^{14}C% of dose incorporated at end of perfusion in:	
				Fibrin	Trans-ferrin 1	Albumin	Plasma proteins	Liver
	Days of treatment							
87	3'-MDAB 2	100	–	0·877	0·370	0·426	8	37
91	18	82	98	1·890	0·912	1·380	25	57
76	28	71	–	0·908	0·746	0·760	–	–
84	32	90–100	100	3·030	1·560	1·250	24	–
89	41	62	60	0·824	0·662	0·707	13	–
98	DMN 2	100	150	1·770	1·010	0·691	14	37
97	19	87	103	1·340	0·642	0·497	15	38
93	44	98	110	0·765	0·798	0·437	8·4	26
94	59	94	112	0·527	0·443	0·173	4·7	18·5
95	66	88	69	0·601	0·677	0·387	7·0	15
	Normal livers (average values)	100	100	1·43*	0·645*	1·35*	23	31†

The fibrin specific activities have been corrected to allow for differences in the fibrinogen concentration in the perfusing blood. For this purpose a concentration of 3·4 mg/ml plasma has been taken. Similarly the specific activities of all three proteins have been corrected to an assumed standard volume of plasma of 30 ml.

All livers were perfused for 5 hr with blood and then with 20 ml 0·9% NaCl. In normals thus treated liver weight is 3·0% of body weight.

*See Table 2 for individual values.

†Average of 86 and 10.

of the three plasma proteins are recorded as observed except for correction for the amount of each present during the perfusion (see below) in Tables 2 and 3. In Table 2 the average specific activity values for the livers from the normal and protein free diet rats are given, corrected for liver weight. The relative weights of those liver donors which were fed 3'-MDAB and DMN are also given in Table 3. Since with abnormal livers the absolute specific activities of the circulating plasma proteins will reflect not only the altered rates of synthesis actually taking place but also any change in the precursor specific activities which has been concurrently brought about, the specific activities observed with these livers have also been expressed relative to the specific activity of albumin found in the same experiment (cf. Table 4). An indication of the importance of these changes in the case of the livers from rats kept on the protein free diet can be seen from Table 1. Despite the absence of similar data for the carcinogen treated livers considerable changes in precursor specific activity must presumably again be assumed.

Corrections for plasma protein pool size during perfusion — The specific activities shown in Tables 2–4, have been corrected for the volume and plasma protein concentration of the blood used in each perfusion. Thus the values finally arrived at are those which would have been obtained assuming the presence of 30 ml of plasma containing 7% total plasma protein and 3·4 mg/ml fibrinogen.

The amount of fibrinogen present varied because of the origin of the blood from either male or female rats. As the concentration of fibrinogen was not measured in each experiment the amount present was assumed to be that found by the method of Ellis and Stransky [18] for the plasma of male or female rats similar to those used as blood donors. The blood used for 10 of the perfusions was obtained from male rats weighing more than 400 g each. In three other perfusions the blood was from females weighing approximately 200 g. For the remaining perfusions blood from male rats weighing 300–400 g was used. The blood from the male rats of over 400 g contained 4·6 mg and that from the females only 2·1 mg fibrinogen per ml plasma. In addition fibrin was isolated from the blood at the end of each perfusion and weighed. For details of the clotting procedure used see page 00. In four perfusions when blood from the large male rats was used the amounts of fibrin thus obtained ranged from 66–72% of the amounts shown to be present originally by the method of Ellis and

Table 4. Relative ^{14}C specific activities of plasma proteins isolated at end of liver perfusion

	Specific activities as % of albumin specific activity in the same plasma											
Perf. No.		Fibrin	Tr 1*	Perf. No.		Fibrin	Tr 1*	Perf. No.		Fibrin	Tr 1*	Perf. No.		Fibrin	Tr 1*
75	Normals	75	58	87	3'-MDAB	206	87	83	Protein free diet	251	88	98	DMN	255	145
78		126	57	91		137	66	85		206	92	97		271	125
86		77	42	76		119	98	02		261	113	93		175	182
09		158	51	84		243	125	05		94	54	94		304	265
06		102	–	89		116	93	08		164	60	95		155	175
Average		108	52			164	94			195	81			232	178
SEM		35·0	3·7			25·5	9·5			30·6	11·1			28·6	23–9
±		32·4%	7·1%			15·6%	10·1%			15·7%	13·7%			12·3%	13·4%
				Average as % of normal		152	181			180	156			215	343
Significance of difference from result with normals P<							0·01				0·1†				0·01

Correction for pool sizes are as in Table 3
*Transferrin 1.
†P=0·06.

Stransky [18]. Very similar yields (56–77%) were obtained in three other perfusions in which blood from female rats was used.

Purity of isolated transferrins — Electrophoresis in starch gel of approximately 1 mg amounts of each isolated transferrin indicated variable degrees of contamination. Photographs of the stained gels thus obtained from certain of the perfusions are shown in Fig. 1.

The transferrins isolated from perfusions No. 97 and 98 (DMN treated rats) were also examined. Transferrin 1 from both showed only minor heterogeneity similar to that found with transferrin 1 of perfusion No. 87.

Since previous work [16] had shown the difficulty of obtaining transferrin free from traces of substances containing haematin when haemolysed plasma is used as starting material, transferrin 1 from perfusion No. 86 was examined for such substances. This was done by measurement of light absorption at 557 mμ after conversion of haematin compounds to pyridine haemochromogens. The value thus obtained showed less than 1% contamination with such proteins. This calculation is based on a M mol extinction coefficient at 557 mμ of 31·4 and assumes the presence of 4 molecules of haem in a molecule of 67,000 wt.

Contamination with substances of differing ^{14}C leucine specific activity was further investigated by chromatography of a pooled sample of transferrin 1 on a column of carboxymethyl–Sephadex C50 (cf. p. *20*). The transferrin emerged as a single sharp peak with ^{14}C leucine specific activity 91·5% of that found before treatment.

Possible contamination with liver proteins was investigated by means of an anti-serum which had been made by injection of a rat liver homogenate into rabbits [20]. This anti-serum when absorbed with rat plasma and used on an Ouchterlony plate gave 3 lines with plasma which had been used for perfusion of a rat liver. On the other hand no line appeared with transferrin 1 (0·62 mg) when this had been isolated from blood used for perfusion of a normal liver. Transferrin 1 (0·38 mg) obtained from blood used in perfusion No. 02 (protein free diet liver donor) was also tested with the same result.

Purity of isolated albumin — This was examined both by electrophoresis in starch gel and by means of gel diffusion using two suitable anti-sera. By the electrophoresis, the albumin isolated from plasma used for perfusion of a normal liver gave a single main band together with a minor component of slightly lower mobility. Charlwood [21], who made a detailed investigation of rat albumin prepared. in this way, reported a similar inhomogeneity. Similar results were obtained when the albumin was obtained from plasmas used with the livers of rats which had received the three abnormal diets.

The samples of albumin obtained in perfusion No. 10 at 4, 6·8 and 11 hr after commencement were tested with the two anti-sera. These were an anti-serum against rat liver similar to that used by Gordon [20] and an anti-serum raised in rabbits against a fraction consisting mainly of albumin which had been obtained by electrophoresis of rat plasma. With this anti-serum all 4 samples gave one very strong and one weak line except for the 11 hr sample which gave in addition a second very weak line.

With the anti-serum against rat liver only the 11 and the 8 hr samples were positive, the former giving a single weak line and the latter a just detectable diffuse zone.

Specific activities of liver and plasma free ^{14}C leucine — Information on the specific activities of the precursor ^{14}C leucine in livers after short periods of perfusion is given in Table 1. Presumably because of the smaller pool of free leucine (40% of that present in the normal livers) in the liver from a rat that had received the protein free diet, its specific activity at 20 min after addition of the radioactivity was found to be 2·08 times that obtained for a normal liver. Although at 36 min the specific activities were equal the initial high value for the specific activity in the liver from the dieted animal may be important in relation to the specific activities of plasma proteins labelled by livers of this type.

Doubtless the observed free ^{14}C leucine specific activities on which Table 1 is based do not correspond exactly with the specific activities at the site of synethesis of the plasma proteins. Thus whereas ^{14}C leucine in peptide linkage or other combined form as well as free ^{14}C leucine was included in the estimate of total radioactivity, leucine in combined form will not have contributed to the estimate of total leucine present. In addition the existence of different proportions of parenchymal and other cells in the different livers must limit accuracy.

Specific activities of plasma proteins after various times of perfusion — The livers from two normal rats were perfused for 7 hr and samples of fibrin and albumin were isolated every hour starting at 4 hr after addition of the ^{14}C leucine. As shown in Fig. 2, transferrin 1 was also isolated at 5 and 7 hr. These three plasma

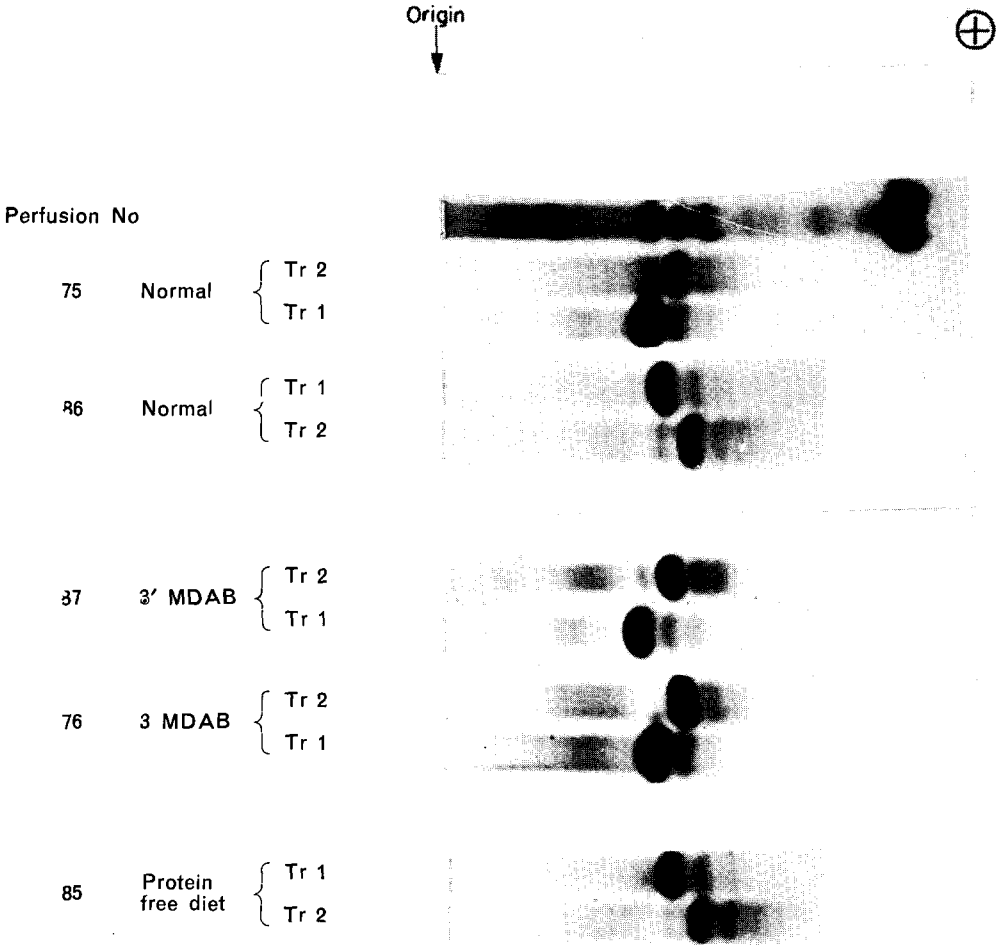

Fig. 1. *Homogeneity of isolated transferrin* 1 *as indicated by electrophoresis in starch gel. Samples of transferrin* 2 [16] *also isolated in these experiments, have been included in each gel. The bands thus obtained indicate that each sample of transferrin* 1 *is contaminated by traces of transferrin* 2. *Slight traces of slower running material are also present, especially in the sample of transferrin* 1 *from perfusion No* 76.

(to face p. 24)

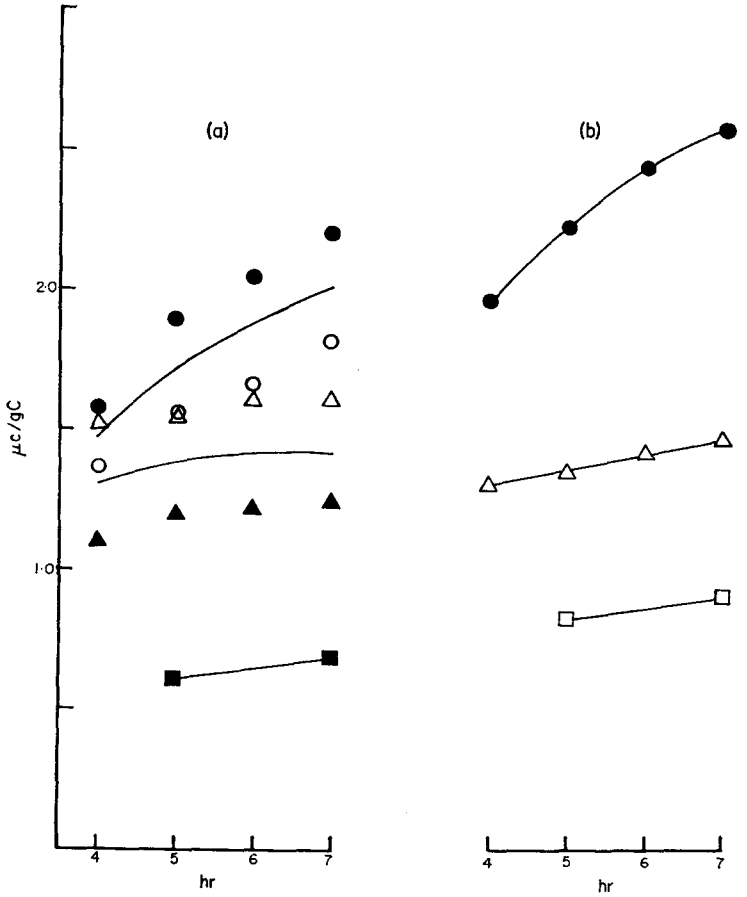

Fig. 2a. ^{14}C *leucine specific activities of fibrin, albumin and transferrin* 1, *after different times of perfusion of the livers of two normal rats* ○ *=fibrin, and* △ *=albumin from perfusion No* 06, ● *=fibrin,* ▲ *= albumin, and* ■ *=transferrin* 1 *from perfusion No* 09 2·5 μc *of* ^{14}C *leucine was added at zero time.*
Fig. 2b. ^{14}C *leucine specific activities of the same plasma proteins after different times of perfusion of the liver of a rat which had received the protein free diet,* ● *=fibrin,* △ *=albumin, and* □ *=transferrin* 1 *from perfusion No* 08.

proteins were also isolated at similar times during perfusion No. 08. In this experiment the liver was from a rat which had been kept for 73 days on the protein free diet. In addition one normal liver (perfusion No. 10) was perfused for 11 hr. The specific activities of fibrin and albumin isolated at 2 hr intervals are shown in Fig. 3. Evidently at 5 hr the specific activities of albumin and transferrin but not fibrin have nearly reached their final values. The further increase of 12% in the specific activities of albumin which occurred after this period (Fig. 3) may well be the result of the traces of liver proteins which were found on testing with the anti-sera (cf. p. *24*). As shown in Fig. 2, the shape of the curves relating specific activity to time perfused, for the liver of a rat which had received the protein free diet are similar to those obtained with normal livers, thus the ratios of the specific activities of transferrin 1 and albumin after 5 hr perfusion of these livers may be compared with the same ratios obtained with the normal livers.

Protein free diet — After 7–10 weeks on this diet the average incorporation of ^{14}C leucine into transferrin 1 and fibrin by the perfused liver was found to be above the values obtained for these two proteins using normal livers.

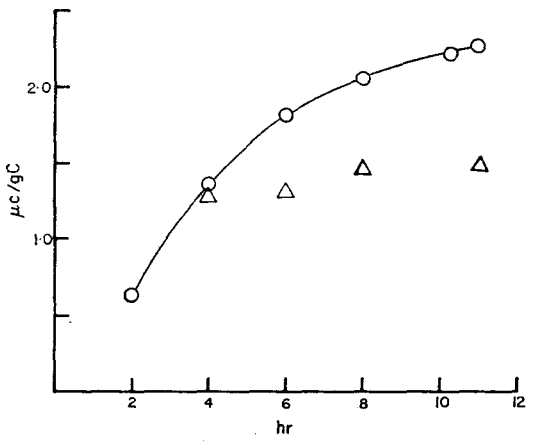

Fig. 3. Perfusion for 11 *hr* ^{14}C *leucine specific activities of fibrin and albumin after different times of perfusion of the liver of a normal rat.* ○ *=fibrin, and* △ *=albumin.*

The results obtained by perfusions of five livers of each kind are given in Table 2 together with the SEMs. The average specific activity of albumin obtained with the treated livers was reduced by 15% when expressed directly but was increased when expressed per g liver. These changes were less than the SEM of the total of 10 experiments. The average specific activities of transferrin 1 and fibrin expressed as a percentage of the specific activity of albumin found in the same experiment are given in Table 4.

3′–MDAB diet — As shown in Table 3 the specific activities of the three proteins isolated from perfusion of livers from rats which had received 3′–MDAB varied greatly from experiment to experiment. However, when expressed in relation to the specific activity of the albumin isolated from the same experiment the results were much less variable (Table 4). On this basis the average relative specific activity of transferrin 1 given by the treated livers is 181% of that obtained with normal livers ($P<0{\cdot}01$, cf. Table 4). Although the corresponding average value for fibrin is 152% of that given by the normals the result is not significant P^{W} 0·1 owing to the rather considerable scatter of the fibrin values given by the normal livers. Transferrin with the highest specific activity was given by a liver No. 84 from a rat which had received the 3′–MDAB diet for 32 days. In perfusion No. 76 although the diet had been fed for a slightly shorter period the animal had already lost approximately 30% of its initial weight; both the transferrin and the albumin specific activities were found to be lower than in experiment No. 84.

DMN diet — The rats kept on this diet rapidly ceased growth and then lost a small and variable percentage of their weight. As shown in Table 3, concurrently with some loss of body weight the liver weights increased and with the sole exception of the rat kept for the longest period (66 days) on the diet, were all above normal when perfused. The specific activities of the albumin isolated in all the experiments with DMN treated livers were always well below those obtained when normal livers were used. Not surprisingly the lowest albumin specific activities were found when the liver donors had been dieted for the longer periods (Fig. 4).

The values obtained with DMN treated livers for the specific activity of fibrin and transferrin 1, considered in relation to the albumin in the same experiment are shown in Table 4. Evidently this treatment led to a large increase in the average relative specific activity of both transferrin 1 and fibrin. These increases to 343% and 215% of normal res-

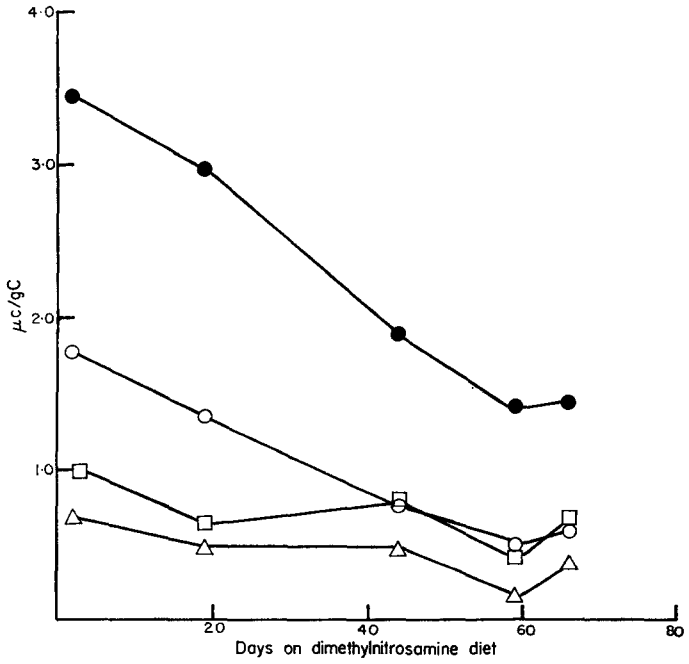

Fig. 4. ^{14}C *leucine specific activities of albumin, transferrin* 1 *and liver protein after* 5 *hr perfusion of livers from rats kept on a diet containing dimethylnitrosamine for different periods of time.* ○ *= fibrin,* △ *= albumin,* □ *= transferrin, and* ● *= liver protein. The values for the three plasma proteins have been corrected only for the amounts of each protein present during perfusion (cf. p. 22). The* ^{14}C *specific activities of the samples of liver have been corrected for liver size using the values for liver wet weight given in Table* 3. *The specific activity shown for liver at* 2 *days is probably too high because the wet weight of this liver (Perfusion No.* 98) *was well above normal.*

pectively were greater than the similar increases found for 3′–MDAB and for the protein free diet.

DISCUSSION

The primary aim of the present work has been to investigate the effects of certain abnormal treatments which when applied, to the liver donor, alter the rates of synthesis of the plasma proteins. Since in practice the incorporation of ^{14}C leucine into each plasma protein after five hours perfusion has usually been measured, it is necessary to consider to what degree such data are proportional to the relative and absolute rates of synthesis of each protein.

For the relative rates of synthesis of the plasma proteins by both normal and abnormal livers to be proportional to the observed specific activities of these proteins in the perfusing plasma the following assumptions would seem to be necessary:

1. The proteins are isolated after all the newly formed ^{14}C leucine labelled proteins have had time to leave the liver cells.
2. All the proteins are formed from the same intracellular pool of free amino acids.
3. An insignificant proportion of each of the newly formed proteins is catabolised during the course of the experiment.
4. Suitable corrections are applied to allow for the different amounts of each protein present in the perfusing plasma.

Investigation of the rates of catabolism of albumin [12] and transferrin [22] by the perfused liver has shown that assumption [3] is likely to be true at least after the newly formed proteins have left the liver cell. On the other hand differential catabolism of newly formed protein, before secretion into the perfusing plasma, if found to occur, could explain changes in the specific activity such as those found in the present work.

After a single addition of a radioactive amino acid, in this work ^{14}C leucine, to a liver perfusion system the specific activity of the intracellular free amino acid pool will pass through a maximum and then decline rather rapidly (cf. p. *24*). As a result ^{4}C leucine containing plasma proteins of at first rapidly rising and then declining specific activity will leave the liver cells and accumulate in the circulating plasma.

Because the specific activity of the liver free amino acid falls rapidly, the experimental period actually used is sufficiently long for almost all the labelled protein molecules to have left the liver. That this has occurred is shown in Figs. 2 and 3 in which the plasma protein specific activities are plotted against the time of perfusion. As shown the specific activities of albumin and transferrin are almost maximal after 5 hr whereas this is true for fibrinogen only after 11 hr. That these maxima are not simply due to termination of the ability of the liver to synthesize plasma proteins was shown by Miller, Bly, Watson, and Bale [23] who added a second dose of ^{14}C lysine during perfusion of a rat liver and obtained a renewed increase in plasma specific activity.

Once all the labelled protein molecules have left the liver, factors such as the size of the intracellular pool of each protein and the rate of secretion from the liver need no longer be taken into consideration in relation to the synthesis rates if these latter are estimated from the observed maximum specific activities of each protein in the perfusing plasma. When corrected for the relevant plasma pool sizes these specific activities will be proportional to the rates of synthesis.

Protein free diet — The concentration of certain of the plasma proteins after prolonged feeding of a protein free diet has been investigated by Weiner and Godfrey [27]. After 63 days on this diet when the concentration of the albumin had fallen to 48% of normal that of the globulins was much less affected. In particular the β-globulin fraction which will have contained the transferrin was least changed (79% of normal at the same time). The catabolic and synthesis rates of albumin in rats maintained on a protein free diet have been investigated by Freeman and Gordon [10]. After 12 days on the diet the rate of synthesis was found to have fallen to just under half the rate observed when the rats were kept on a normal diet. In the present work the livers used for perfusion were from the same strain of rats as were used by Freeman and Gordon [11] but after feeding the protein free diet for a longer period. Thus presumably in *vivo*, before transfer to the perfusion circuit, the rate of synthesis of albumin by the livers of these rats will have been well below that of a normal liver. On perfusion of such a liver, assuming no change in the precursor specific activity, the reduced synthesis rate might have been expected to lead to a greatly reduced absolute ^{14}C leucine specific activity of the albumin. In fact, however, the average specific activity of the albumin after 5 hr perfusion of livers from the rats which had received the protein free diet was 85% of that

obtained by perfusion of livers from normal rats (Table 2).

The observed equal or almost equal specific activities of the albumin in these experiments can most simply be explained as being the result of two opposing effects. Thus the effect of the increased intracellular ^{14}C leucine specific activity observed initially during perfusion of a liver of a protein deprived rat (Table 1) is likely to have been cancelled out by continuation during the perfusion of the low rate of albumin synthesis known to occur *in vivo*.

As shown in Table 4 the average ratio of the specific activities of transferrin 1 and albumin after perfusion of livers from protein deprived rats is 156% of that obtained with normal livers. This increase presumably results directly from the increased specific activity of the precursor and does not represent any increase in synthesis rate of this protein. On this interpretation the decreased synthesis rate of albumin known to occur *in vivo* has continued during perfusion. Similarly a more normal synthesis rate of transferrin which seems probable *in vivo* in animals on this diet [24] will have continued during perfusion.

3′–MDAB and DMN — Examination of the serum of rats bearing liver tumours due to 3′–MDAB [25] has revealed that at this stage after 7–14 months of dieting the concentration of albumin is normal whereas that of β-globulin is increased to an average of 125% of normal. In *in vitro* systems the effects of liver carcinogens have been found to include both inhibition and stimulation of protein synthesis. Thus with rat liver slices Arrhenius and Hultin [26] found inhibition after treatment with 2 aminofluorene. On the other hand the same authors [27] using liver microsomes found stimulation of incorporation of ^{14}C valine by 2 aminofluorene at 9–48 hr. In recent work with cell free systems of liver from rats treated *in vivo* with 3′–MDAB, Hawtrey, Schrirren and Dijkstra [28] observed 300–400% increases in incorporation of ^{14}C leucine. Unfortunately in these experiments no distinction could be made between incorporation of ^{14}C leucine into liver proteins and such plasma proteins as might be synthesised under the conditions chosen.

Incorporation of 6–^{14}C lysine and 2–^{14}C histidine into total plasma proteins during perfusion of rat livers [3, 4] was increased after prolonged feeding with 3′–MDAB. These livers also produced less $^{14}CO_2$ and less urea and had higher intracellular free amino acid concentrations than did those from normal rats after the same period of perfusion. Perhaps because in the present experiments 3′–MDAB was fed for shorter periods incorporation of ^{14}C leucine into the total plasma proteins after 5 hr perfusion was either normal or reduced (Table 3).

When DMN was fed incorporation of ^{14}C leucine into the whole plasma and liver decreased with the time of dieting of the liver donor. As shown in Fig. 4, the specific activities of albumin transferrin 1 and liver protein all showed the same trend. As a result primarily of the marked inhibition of ^{14}C leucine incorporation into albumin brought about by the DMN the specific activity of the transferrin 1 relative to that of the albumin in the same plasma was increased to 343% ($P<0{\cdot}01$) of that with normal livers. With 3′–MDAB, incorporation into albumin was normal in two experiments and well below normal in three other experiments. With 3′–MDAB as with DMN, for however long the liver donor had received the diet the ratios of the ^{14}C leucine specific activities of transferrin and albumin were always significantly increased ($P<0{\cdot}01$). The decreased absolute or relative specific activity of albumin observed in all these experiments with carcinogens is more marked than the similar change observed with the livers from rats which had received the protein free diet.

The mechanism by which these different treatments each may lead to relative inhibition of albumin synthesis evidently requires further investigation. At present it is sufficient to note that examination by means of the electron microscope of livers from rats which have been subjected to starvation and to dieting with 3′–MDAB, reveals that conditions similar to those used in the present work lead to livers with fragmentary endoplasmic reticulum from which most of the RNP granules have been detached [29, 30].

A possible explanation of the present findings would be that treatment with both the carcinogens leads to subcellular structural changes such that the free ^{14}C leucine specific activity becomes abnormally variable in different parts of the liver. Depending on chance factors the average free ^{14}C leucine specific activity at the sites of synthesis of the plasma proteins is increased to a very variable degree or occasionally as in the case of perfusions No. 87 and 89 with 3′–MDAB, may remain normal. Superimposed on this change is an inhibition of albumin synthesis which at least with DMN increases with the time of dieting and is always sufficient to more than overcome the change in ^{14}C leucine incorporation due to any increase in the precursor specific activity.

The present finding that the relative specific activity of transferrin to albumin is increased both by previous feeding of the protein free diet and by diets containing 3'–MDAB or DMN suggests the possibility that a common mechanism may be involved. Evidently two alternatives should be considered. If all the plasma proteins which originate from the liver are in fact synthesised simultaneously in all the parenchymal cells then the three treatments probably affect the ratios of the synthesis rates by mechanisms operating at the same time and in the same way in each cell. Alternatively if only some of the parenchymal cells are active at any given time with respect to the synthesis of each of the plasma proteins as is suggested by the work of Hamashima, Harter, and Coons [31] then the different treatments may alter the synthesis rates by diverting cells towards the synthesis of one plasma protein and away from that of another or by activating previously quiescent cells. Further work will be required to enable a choice to be made between these alternatives. Possibly further measurements of the differentital incorporation of ^{14}C leucine into the various plasma proteins by the perfused liver may assist in this aim. In this regard it is notable that the changes brought about in the liver by all the three treatments applied in the present experiments to the liver donors, appear to be sufficiently stable to be studied in the liver perfusion system.

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RESUME

1. *Des foies de rats normaux et de rats traités au 3'MDAB et au DMN, ainsi que de rats soumis à un régime sans protéines durant 7 à 10 semaines, ont été perfusés, et l'incorporation de leucine C^{14} dans certaines protéines du plasma a été mesurée.*

2. *L'albumine, la transferrine et la fibrine ont été isolées du sang perfusé, à différents moments, après l'addition de leucine C^{14}. Alors qu'après 5 heures, les activités spécifiques de l'albumine et de la transferrine étaient presque maximales, l'activité spécifique de la fibrine n'avait atteint que les deux tiers de sa valeur totale.*

3. *Après 5 heures, l'activité spécifique moyenne de la transferrine, isolée de la perfusion de foies de rats qui avaient été soumis à un régime sans protéines, était augmentée en comparaison de celle des foies normaux. Cette augmentation peut être comparée à l'activité spécifique de l'albumine qui ne change pas de façon significative.*

4. *Lorsqu'on exprime l'activité spécifique de la transferrine par rapport à celle de l'albumine produite par le même foie, on constate que, pour des foies de rats soumis à un régime sans protéines, cette activité spécifique de la transferrine atteint* $156 \pm 21{,}4\%$ *de la valeur trouvée pour des foies normaux. Exprimée ce la même façon, l'activité spécifique de la transferrine atteint, pour les foies d'animaux traités au 3'MDAB,* $181 \pm 18{,}3\%$, *et pour les foies d'animaux traités au DMN,* $343 \pm 46\%$ *des valeurs de contrôle.*

5. *L'estimation de l'activité spécifique de la leucine C^{14} libre, durant la perfusion de foies de rats soumis prélablement à un régime sans protéines, suggère que la diminution du taux de synthèse de l'albumine, que l'on sait se produire chez de tels animaux "in vivo", continue dans le foie pendant la perfusion.*

SUMMARY

1. *Livers from normal, 3'-MDAB, and DMN treated rats and also from rats which had been fed on a protein free diet for 7–10 weeks have been perfused and incorporation of ^{14}C leucine into certain of the plasma proteins and into the livers has been measured.*

2. *Albumin, transferrin and fibrin were isolated from the perfusing blood at various times after the addition of the ^{14}C leucine. Whereas at 5 hr the specific activities of albumin and transferrin were found to be almost maximum at this time the specific activity of fibrin had only reached two thirds of its final value.*

3. *At 5 hr the average specific activity of transferrin isolated from the perfusion of livers of rats which had been kept on the protein free diet was increased compared with that obtained*

when normal livers were used. This increase may be compared with the specific activity of the albumin which did not change significantly.

4. *Expressed in relation to the specific activity of albumin given by the same liver the specific activity of transferrin obtained from the livers of rats which had received the protein free diet was* $156 \pm 21 \cdot 4\%$ *of that when normal livers were used. Similarly expressed the transferrin from 3'-MDAB treated livers was* $181 \pm 18 \cdot 3\%$ *and that from DMN treated livers* $343 \pm 46\%$ *of that obtained with normal livers.*

5. *Estimates of the specific activity of free* ^{14}C *leucine during perfusion of the livers of rats previously subjected to the protein free diet suggest that the decreased rate of albumin synthesis known to occur in such animals* in vivo *continues in the liver during perfusion.*

ZUSAMMENFASSUNG

1. *Die Lebern normaler und mit 3'-MDAB oder mit DMN vorbehandelter Ratten ebenso wie Lebern stammend von Ratten, welche während 7 bis 10 Wochen eiweissfrei ernährt worden waren, wurden perfundiert und die Einverleibung von mit* C^{14} *markiertem Leucin in gewisse Plasmaproteine und in die Lebern bestimmt.*

2. *Albumin, Transferrin und Fibrin wurden aus dem Perfusionsblut in bestimmten Zeitabständen nach der Zugabe von* C^{14}*-Leucin isoliert. Nach 5 Stunden waren die spezifischen Aktivitäten des Albumins und des Transferrins bereits an ihrem Höhepunkt, während zu diesem Zeitpunkt die des Fibrins erst zwei Drittel ihres Endwertes erreichte.*

3. *Nach 5 Stunden war die Durchschnittsaktivität des Transferrins, welches aus dem Leberperfusionsblut der eiweissfrei ernährten Ratten stammte, erhöht, verglichen mit dem Blut normaler Lebern. Im Gegensatz hierzu war die spezifische Aktivität des Albumins in diesen beiden Versuchsanordnungen nicht einwandfrei verschieden.*

4. *Wenn man die spezifische Aktivität des Transferrins mit der spezifischen Aktivität des Albumins derselben Leber vergleicht, so ist diese Relation* $156 \pm 21{,}4\%$ *in dem Falle der eiweissfrei ernährten Ratten verglichen mit den normalen Lebern. Ebenso ausgedrückt beträgt diese Relation für das Transferrin von mit 3'-MDAB behandelten Lebern* $181 \pm 18{,}3\%$ *für das Transferrin von mit DMN behandelten Lebern* $343 \pm 46\%$*, verglichen mit den Werten von normalen Lebern.*

5. *Die Bestimmungen der spezifischen Aktivität von freiem* C^{14}*-Leucin in dem Leberperfusionsblut von eiweissfrei ernährten Tieren lassen den Schluss zu, dass die verminderte Albuminsynthese, welche bekannterwiese in diesen Tieren in vivo stattfindet, auch in den perfundierten Lebernfort besteht.*

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Quantitative Evaluation of the Radiosensitivity of Cells of a Transplantable Rhabdomyosarcoma in the Rat

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INTRODUCTION

ONE OF the characteristic properties of tumour cells is their capacity for unlimited proliferation which to a large extent determines the clinical course of the disease. It is generally assumed that single tumour cells can generate new tumour nodules or metastases when they are embedded in a suitable site. If the therapeutist is capable of arresting the proliferation of all the tumour cells, the disease will be cured regardless of whether the tumour cells will differentiate or vanish after degeneration.

Irradiation of cells with ionizing radiation may result in loss of their capacity for unlimited proliferation. The relationship between X-ray dose and the percentage of permanently inactivated cells of mammalian origin was first investigated by Puck and Marcus [1]. They used tissue cultures and a special technique which allows individual cells to proliferate and form isolated colonies (clones). The cells which give rise to colonies after irradiation have "survived" the irradiation and the term "survival curve" is used for the graphical plot of this dose–effect relationship [1, 2].

The shape of many mammalian cell survival curves for X- or γ-rays is characterized by an initial "shoulder" followed by a straight part if the log survival is plotted against as a function of the dose. The slope of the exponential part may be described by the D_{37}, being the increment of dose required to decrease the surviving fraction to 1/e or 37%. A characterization of the shoulder may be found in the "extrapolation number" (N); this is the point of interception found by extrapolation of the exponential part of the curve to the ordinate.

Numerous papers have been published during recent years dealing with different aspects of survival curves, for instance their relation to the LET of the radiation [3] fractionation of the dose [4], the theoretical implications of the shape of the curve [5].

Of the many papers describing survival curves of animal tumour systems only some will be mentioned here. Hewitt and Wilson [6] were the first to publish a survival curve obtained with a transplantable mouse leukaemia. They employed an assay system which had been described by Hewitt [7] previously, using the calculated cell number which yielded 50% tumour takes as an indication of tumour cell viability. Their dose–survival curve was very similar to the curves obtained by others with cells in tissue culture.

The problem whether a comparable dose–effect relationship also applies to the cells in solid animal tumours has been approached recently by various workers with different systems. Hewitt referred to experiments with a round cell sarcoma [8] which showed a similar response as was obtained with mouse leukaemia cells, when irradiation of the latter

was performed under anoxic conditions. Powers and Tolmach [9] reported a cell survival curve of a solid mouse lymphosarcoma, using the Hewitt assay system. Suit and Shalek [10, 11] irradiated anoxic isotransplants of a C3H mouse mammary carcinoma and calculated the D_{37} from the growth delay after irradiation. They also calculated the D_{37} from the 50% cure dose of spontaneous mammary carcinomas. Clifton and Draper [12] determined the survival curve of a transplantable mouse adenocarcinoma by quantitative transplantation of a crude cell suspension of the tumour. The D_{37} values of the survival curves derived so far from experimental tumours under anoxic conditions show a similarity of D_{37} values which range from 260 to 458 rad of X- or γ-rays. The experiments mentioned by Hewitt [8] indicate a majority of anoxic cells in a solid round-cell sarcoma, while the results obtained by Powers and Tolmach [9] by irradiation of the solid mouse lymphosarcoma seems to suggest the presence of about one per cent anoxic cells in this tumour. So far no information has been published about the proportion of anoxic cells in "radioresistant" tumours.

Our interest was directed towards determining the survival curve of an animal tumour system which should resemble human cancer as much as possible. Improvement of existing cell dispersion techniques [13] allowed reproducible quantitative transplantation of cells obtained from solid tumours so that early in 1963 experiments could be started with a rhabdomyosarcoma of the rat.

This paper deals with experiments designed to determine the cell survival curves of this tumour under three different conditions:

1. Irradiation *in situ* under complete anoxia.
2. Irradiation of a tumour cell suspension equilibrated with air.
3. Irradiation *in vivo* under normal conditions of vascular (oxygen) supply.

MATERIALS AND METHODS

Tumour

The tumour used in this study arose in December 1962 in the mandibula of a rat radiation chimera in our laboratory. Microscopically it was classified as a typical rhabdomyosarcoma; myofibrils and transverse striation could be demonstrated with the phosphotungstic haematoxylin stain in the first tumour generation. The tumour is composed of strap-like myoblasts arranged in interlacing bundles (Fig. 1). The experiments described in this paper were performed with the third to eighteenth generation of this tumour, which is isologous in our WAG/Rij inbred strain of rats.

Transplantation

Transplantation for routine propagation and quantitative assays has been carried out by subcutaneous injection of suspensions of tumour cells. The cell suspensions were made with an improved trypsinization technique [13] and inoculations for assays were made subcutaneously between the ears of 3–4 week old rats (Fig. 2). This site was chosen for reasons of convenience; daily inspection for the appearance of the tumour is greatly simplified and does not necessitate frequent palpation.

The inoculation volume was 0·1 ml, the required number of cells was obtained by dilution in ice-cold Simm's solution before inoculation. The non-irradiated tumour cells were usually inoculated in 7 doses: 1, 4, 10, 40, 100, 400 and 1000 cells, using 10 animals per dose (5 males and 5 females). No difference in tumour growth between the sexes was observed. For the irradiated tumour cells the inoculum was proportionally higher namely inversely proportional to the expected fraction of surviving cells.

From preliminary experiments evidence was obtained that the vast majority of the tumours originating from the smallest inoculations arose

Table 1. *Number of "takes" after inoculation of cell suspensions with or without admixture of heavily irradiated cells*

Inoculum			
Viable cells		Heavily irradiated cells	Number of takes
1000		—	4/5
1000	+	$3{\cdot}3\times10^6$	5/5
100		—	3/5
100	+	$3{\cdot}3\times10^6$	3/5
10		—	2/5
10	+	$3{\cdot}3\times10^6$	0/5

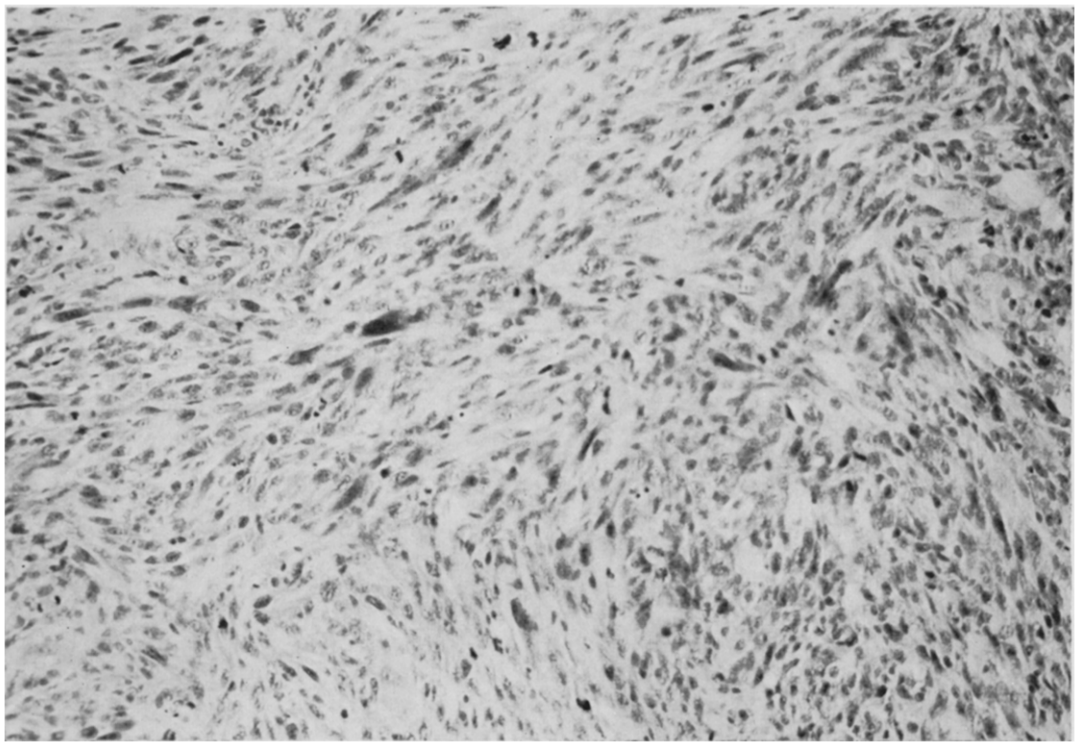

Fig. 1. *Rhabdomyosarcoma BA* 1112. *The tumour is composed of strap-like myoblasts, arranged in interlacing bundles. Haematoxylin–eosin.* × 120.

Fig. 2. *The choice of the subcutaneous tissues of the head of the animal as site of inoculation allows easy inspection of tumour growth.*

(to face p. 34)

within 70–80 days after inoculation. An observation period of 100 days was adopted to take into account possible growth-rate variations.

After high doses of radiation the inoculated cell suspension obviously will consist of few surviving cells mixed with an abundance of non-surviving cells. In the presence of an antigenic difference between the tumour and the host this might influence the growth of the tumour cells and interfere with the assay since the amount of injected antigenic material will vary in different assays [14, 15]. However, no evidence of antigenicity has been observed in this tumour–host system, the number of "takes" observed with injected cell suspensions purposely mixed with large numbers of heavily irradiated cells did not differ significantly from that of controls (Table 1). Similar figures were obtained, when the host-animals were immunized 1 week before inoculation with an intraperitoneal injection of five million heavily irradiated cells, or when animals were immunized by growing tumours that were tied-off 1 week before the test-inoculation. Also the number of takes observed in hosts irradiated with 250 rad of X-rays did not differ significantly from that of unirradiated animals after inoculation of identical numbers of cells.

Irradiation

The irradiations were performed with a General Electric "Maxitron" X-ray unit; 250 kVp, 30 mA, $HVL_{2.1}$ mm Cu, dose rate 40 to 200 rad/min; full backscatter was employed. Depth dose homogeneity was better than 5%. The X-ray dose was measured with a Baldwin "Ionex" dosimeter. The ionisation chamber was placed in a fixed position in the X-ray beam, either in juxtaposition to the tumour or in a position analogous to the tumour(s).

Experimental procedures

The first series of experiments was performed to determine the dose survival curve of anoxic cells of this tumour according to Hewitt's method [6]. The animal was killed 10 min before irradiation in order to let the tumour cells consume all available oxygen in the tumour tissue. After irradiation the tumour was excised, the cell suspension prepared and appropriate cell numbers were inoculated into the recipient animals.

The second set of experiments dealt with the survival curve of well–oxygenated cells. Because it is unlikely that in solid tumour tissue a condition of adequate oxygenation for all cells can be reached, it was decided to perform the irradiation on tumour cells in suspension. For that purpose cell suspensions containing about seventy million cells/ml were irradiated under full aeration. Preliminary experiments showed that when such a cell suspension was irradiated in a flat petri dish, oxygenation was not adequately maintained. This was probably due to the fact that diffusion of oxygen in the suspension was not sufficient to compensate for oxygen consumption of the cells, leaving some of the lowermost cells of the 2 mm thick layer anoxic. In the procedure finally adopted the cell suspension was irradiated in a "roller tube" type mounting of a plastic tissue culture flask. This flask was rotated slowly along its longitudinal axis, which had a slight angle of inclination. In this way the whole of the suspension was in good equilibrium with the air in the flask, which was continuously flushed by a jet of fresh air entering through a hole in the centre of the stopper. After irradiation the required cell numbers were inoculated into the recipient animals.

The third set of experiments was performed to obtain a survival curve of the tumour when irradiated *in vivo*, with its normally existing blood supply. For these experiments tumours were used which were grown on the head of the animal as described before. Only tumours weighing less than 1 g were used. A total of 150 of these tumours were irradiated in these experiments; their average weight was 0·4 g. Care was taken that the blood supply of the tumour was unchanged during the irradiation; for that reason the animals were not anaesthetized, but were restrained either by fixation of the legs with adhesive tape, or by irradiating them in a well-ventilated perspex restraining box. The field of irradiation was usually limited to the head and neck area of the animal. They were allowed to move their head freely within the area where the X-ray dose was uniform within 5%. After irradiation the procedure was similar as described for the anoxic cells. To obtain the three survival curves 84 different experiments were performed; 48 irradiation and 36 control experiments, employing a total of 4228 host-animals.

RESULTS

The unirradiated (control) ED_{50} calculated according to the method of Litchfield and Wilcoxon [16] was in early experiments equal to 40 cells (Fig. 3). This means that a calculated number of 40 cells produces a tumour "take" in 50% of the recipient animals within the observation period of 100 days. The ED_{50}

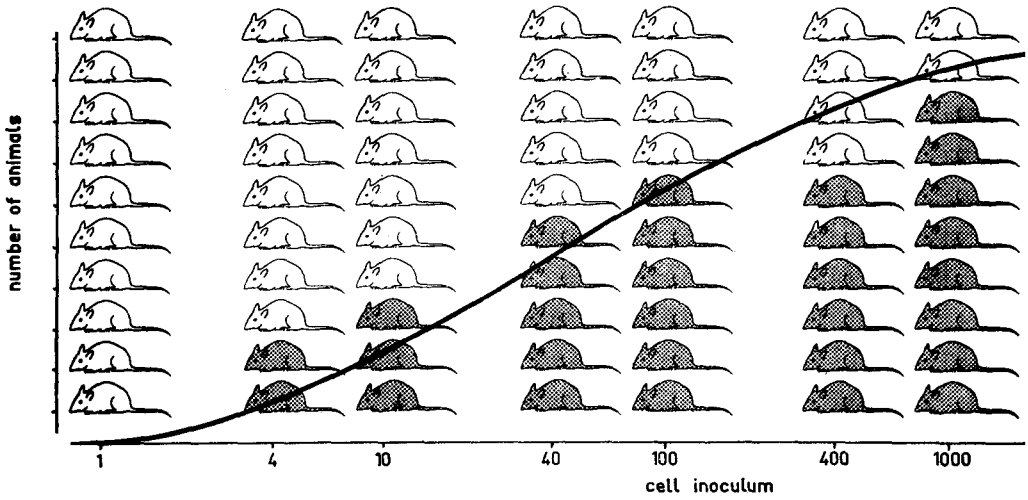

Fig. 3. Example of a "cell titration" experiment. The cell inoculum is plotted on the abscissa on a log scale, the number of animals which has developed a tumour at the site of inoculation is presented schematically (hatched).

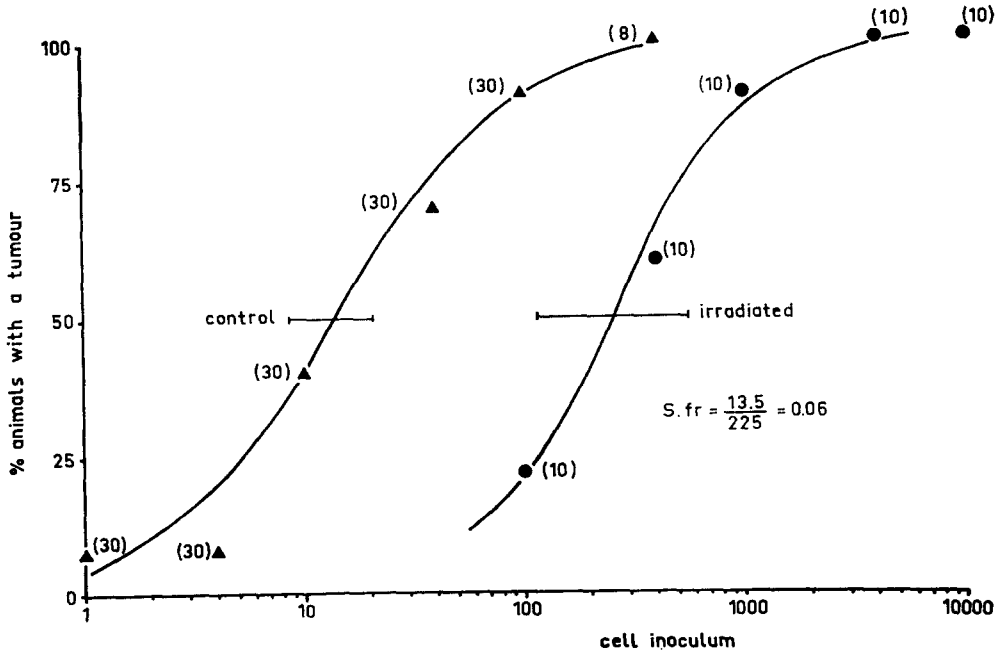

Fig. 4. Example of the calculation of the surviving fraction; (the "N_2" point of Fig. 6). The surviving fraction (s.fr.) is calculated from the expression:

$$S.fr. = \frac{ED_{50} \text{ control tumour cells}}{ED_{50} \text{ irradiated tumour cells}}$$

The values between brackets indicate the number of animals per point.

tended to decrease to approximately 10–20 cells about a year after the first passage, later it increased again. The ED_{50} values of the pooled control experiments consisting of 7 consecutive 3-month periods were 40–37–13–11–16–19–80 cells. The calculation method of Litchfield and Wilcoxon allows pooling of the results and calculation of the confidence limits. The surviving fraction (s.fr.) was calculated as follows: (Fig. 4)

$$\text{Surviving fraction} = \frac{ED_{50} \text{ control tumour cells}}{ED_{50} \text{ irradiated tumour cells}}$$

The data obtained by irradiation of the anoxic tumours are plotted in Fig. 5. The control ED_{50} in this series was 32 cells. The curve drawn through these points represents a survival curve with a D_{37} of $\approx$380 rad and an extrapolation number $N \approx 10$. From the experimental data no conclusion can be drawn whether or not the survival curve at high doses is a straight line on the semi-log plot. For reasons of simplicity, the curve is drawn as a straight line.

The D_{37} value obtained from this curve is very close to the value which Hewitt and Wilson [6] obtained with a mouse leukaemia. The extrapolation number and therefore the shape of the shoulder of the curve are likely to be different ($N=2$ for the mouse leukaemia, $N \approx 10$ for the rhabdomyosarcoma). From our data the exact shape of the shoulder of the curve cannot be estimated due to the rather large experimental errors in this region.

The survival curve of the aerated tumour cells is shown in Fig. 6. The control ED_{50} in this group of experiments was 13·5 cells. The D_{37} value is $\approx$120 rad. One point, obtained in an experiment in which the cell suspension was equilibrated with nitrogen instead of air, is shown to be superimposed on the anoxic curve from Fig. 5. This suggests that Hewitt's method

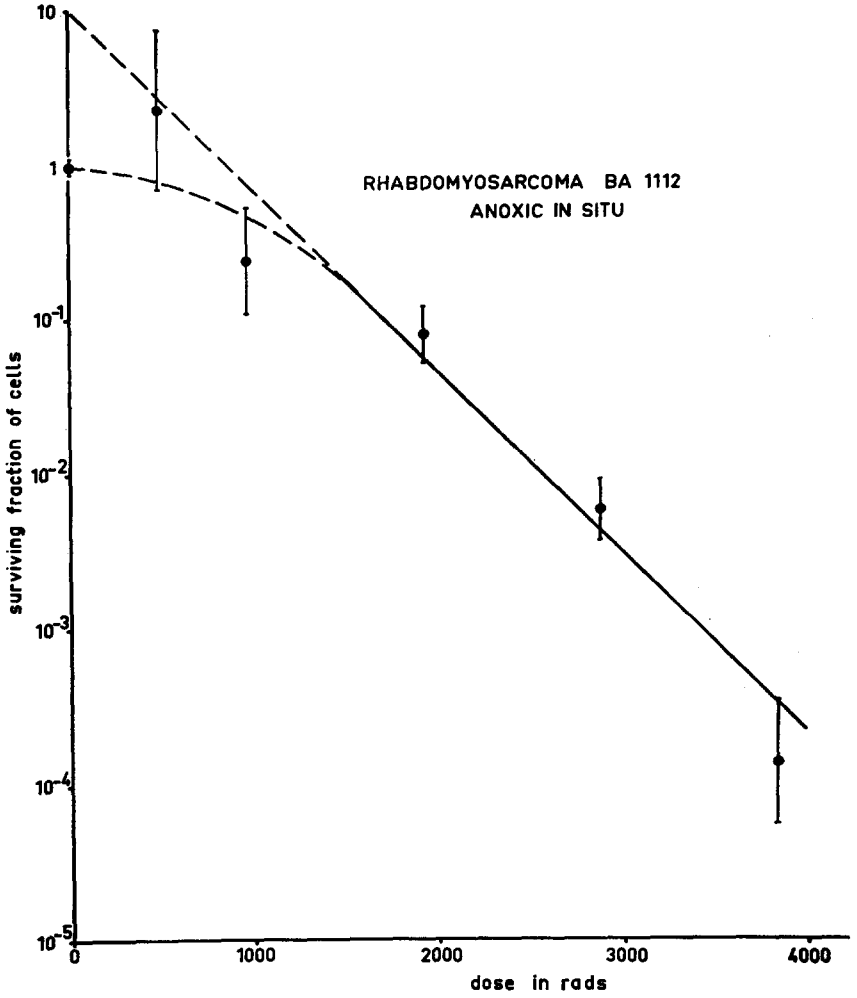

Fig. 5. *Survival curve obtained for anoxic cells of Rhabdomyosarcoma BA* 1112*; irradiation in the dead animal. Curve is fitted by eye.* $D_{37} \approx 380$ *rad.* $N \approx 10$.

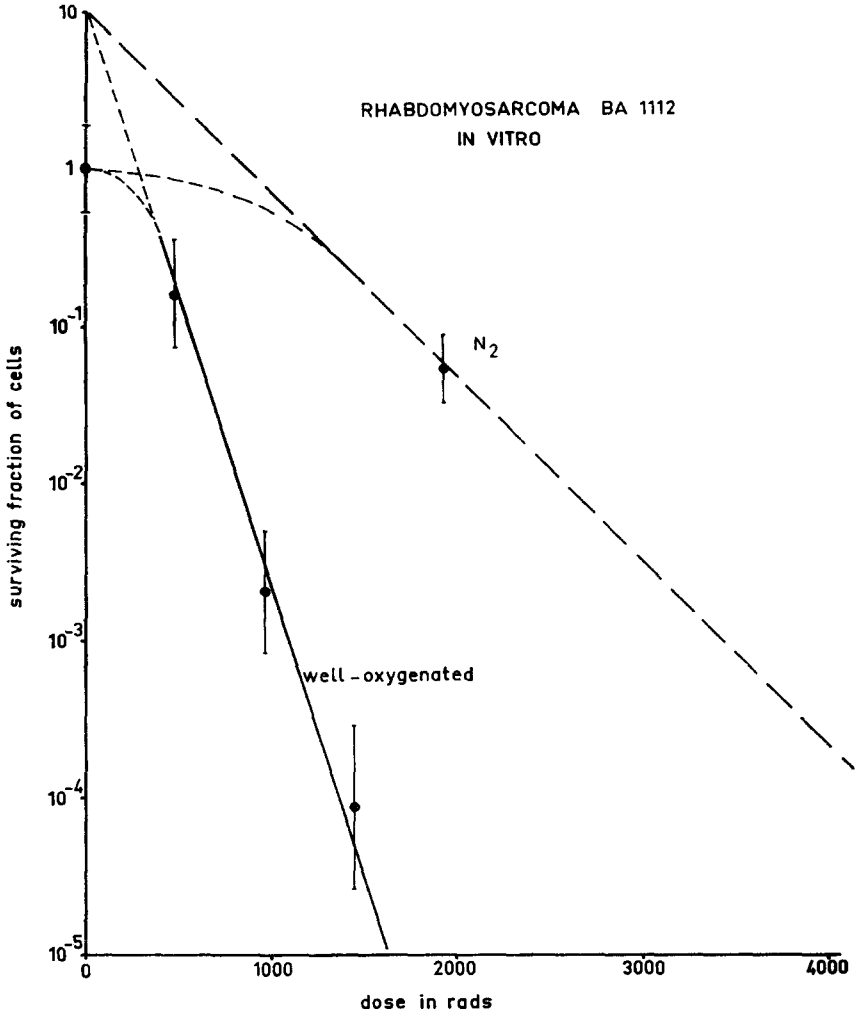

Fig. 6. Survival curve of well-oxygenated cells of Rhabdomyosarcoma BA 1112; irradiation of an aerated cell suspension. Curve fitted by eye. $D_{37} \approx 120$ rad. $N \approx 10$. Note one point ("N_2") irradiated in the same condition but equilibrated with nitrogen, superimposed on (dotted) anoxic curve from Fig. 5.

of inducing anoxia by killing the animal is reliable and that trypsinization of the cells after irradiation did probably not significantly interfere with the survival of the tumour cells. The oxygen enhancement ratio obtained from these data is ≈ 3.

The *in vivo* survival curve, obtained by irradiation of small tumours in the living, unanaesthetized animals with subsequent titration of the tumour cells is shown in Fig. 7. The control ED_{50} in this series was 30 cells. In preliminary experiments, with doses of 480–1920 rad, the values did not differ significantly from those obtained by irradiating the tumours in dead animals (anoxic curve). In further experiments using also higher X-ray doses, the results show that the survival curve differed somewhat from the anoxic curve. The most reasonable interpretation of this finding seems to be that the tumours contained a proportion of anoxic cells. The percentage anoxic cells per point (at each dose level) was calculated from the expression:

$$\text{Percentage anoxic cells} = 100\,\frac{\text{S.fr.}(\textit{vivo}) - \text{s.fr. (well oxygenated)}}{\text{S.fr.(anoxic)} - \text{s.fr. (well oxygenated)}}$$

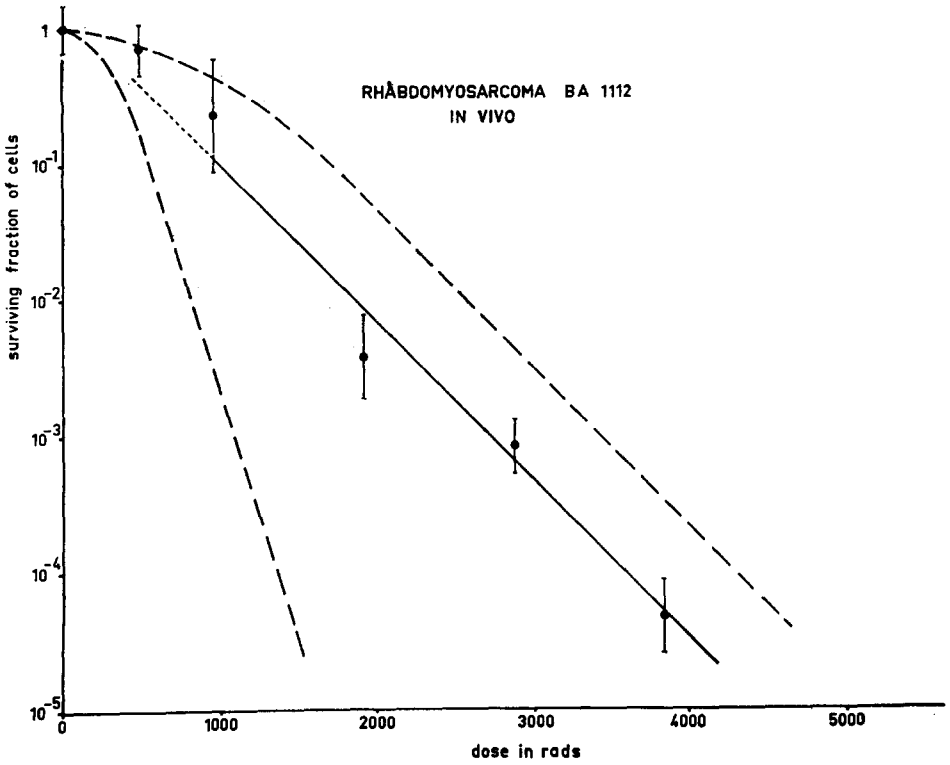

Fig. 7. In vivo *survival curve of cells of Rhabdomyosarcoma BA* 1112; *irradiation of tumours with a weight of less than* 1 *g (mean weight* 0·4 *g, containing* 2×10^8 *cells). Mean deviation of the points from the anoxic curve suggests that* 15% *of the cells are anoxic. Curve is drawn according to Hewitt's* (15) *calculations, assuming* 15% *anoxic cells. Dotted lines represent anoxic- and well-oxygenated curves from Fig.* 5 *and Fig.* 6.

The values scatter from about 10–90%. Because of the uncertainty of the shape of the curves in the shoulder region, the weighed mean was calculated for the three points of the straight part of the curve (doses of 1920, 2880, 3840 rad). As an indication for the weight per point the N value of Litchfield and Wilcoxon [16] was used. According to these calculations the proportion of anoxic cells is about 15%. The survival curve drawn through the plotted *in vivo* points was calculated according to the graph of Hewitt [17] assuming that 15% of the cells are totally anoxic. From these curves it is clear that the surviving fraction of the cells from these small tumours at doses in excess of about 1000 rad is largely determined by the presence of 15% anoxic cells.

The "extrapolation number" of the composite *in vivo* curve is much smaller (15%) than that of the anoxic one, which has also been found by Belli and Andrews [18] in the case of a partially anoxic murine ascites leukaemia.

DISCUSSION

The rhabdomyosarcoma employed in these studies can be considered to be a radioresistant tumour, since a "therapeutic" dose of 30×192 rad of X-rays administered in 6 weeks to a tumour of 15 mm diameter was unable to decrease the size of the tumour. The irradiation merely caused inhibition of the growth of the tumour during that period. When the course of irradiation was terminated at 5760 rad (6000 R) the tumour grew within a month into an ulcerating mass of several cm diameter. Its radioresistance is therefore probably comparable to that of a human rhabdomyosarcoma. It was somewhat surprising that the D_{37} of the survival curve for anoxic cells of this radioresistant tumour turned out to be in the same range as the value obtained by Hewitt and Wilson [19] for a CBA mouse leukaemia, a type of malignancy which is generally assumed to be radiosensitive.

The D_{37} of $\approx$380 rad in the present studies obtained by irradiation under anoxic conditions is comparable with results obtained by others: Hewitt [8], Suit and Shalek [10, 11], Clifton and Draper [12] and Kallman [20] with different solid tumour systems. The D_{37} values for anoxic cells obtained by these workers range from 300 [11] to 458 rad [10]. The cell survival curve of well oxygenated cells of our tumour

allows an estimation of an oxygen-enhancement ratio of ≈3 which is within the usual range [21].

The *in vivo* curve seems to be the most interesting of the three, because it suggests that about 15% of the cells in these small tumours (weight less than 1 g and containing an average number of 2×10^8 cells) can be considered as totally anoxic. This is an over-simplification of the actual situation since there will be a decreasing oxygen gradient from the capillaries into the tissue, leaving many cells at a diminished oxygen concentration. Powers and Tolmach [9] reported a multi-component survival curve for a solid lymphosarcoma, indicating the presence of a mixed population of anoxic and well-oxygenated cells. The percentage anoxic cells in their tumours seems to be less than derived in the present studies with the rhabdomyosarcoma.

Whether cells which are located at unfavourable positions in the tumour tissue will be supplied with some oxygen, will depend in the first place on the efficiency of the vascular system, and secondly on the rate of oxygen consumption of the tissue. The oxygen consumption of the cells of this tumour has been determined [13] and does not differ essentially from the values obtained by others for a variety of tissues [22].

The cause for existence of an anoxic population in these tumours of a limited size should probably be sought in an insufficient vascular supply. Microscopic examination of the tumour tissue indeed suggested that the critical value of intercapillary distances (Thomlinson and Gray [23]) is likely to be exceeded for many locations, especially in the centre of the tumour. Even in a small tumour the percentage surviving cells can be considerably increased by the existence of just a few anoxic foci. The latter can be caused for example by a regionally restricted blood flow, due to increased tissue pressure (caused by tumour growth) [24], or by some abnormalities of the local capillaries which is common to many tumours [25].

The question arises whether any tumour no matter how small, will contain anoxic foci. Smaller tumours than have been used in the present investigations are not very suitable for the preparation of cell suspensions as has also been observed by Powers and Tolmach [9]. Once the survival curve parameters have been determined it seems indicated to investigate the cure-rate of such tumours after local irradiation with different doses of X-rays. If such experiments were to be performed with small tumours of variable size, it might be possible to find a size of the tumour in which there are no anoxic foci.

Another point of interest is the extrapolation number of our rhabdomyosarcoma ($\mathcal{N} \approx 10$). Assuming that the anoxic cells will show full recovery of the Elkind type [26, 27], it will be quite impossible to cure a tumour like this rhabdomyosarcoma by fractionated radiotherapy. This was indeed borne out in preliminary experiments by the poor results obtained with a course of "fractionated radiotherapy" to which this tumour was subjected.

Acknowledgements — I wish to thank Dr. G. W. Barendsen for his assistance concerned with the irradiation set up, Dr. H. Balner for providing the tumour and immunological advice, Dr. M. J. de Vries for the histological interpretations and Dr. J. Kaalen of the Rotterdam Radiotherapeutic Institute for his advice concerning the calculations. I am also indebted to Dr. D. W. van Bekkum for critical advice during the preparation of the manuscript.

RESUME

A l'aide d'une technique améliorée de dispersion cellulaire, des courbes de survie ont pu être obtenues à partir de cellules de rhabdomyosarcome de rat venant d'une tumeur transplantable. Les cellules tumorales irradiées et non irradiées ont été inoculées, selon des dosages différents à des hôtes isologues.

La ED_{50} *des tumeurs non irradiées, calculée selon la méthode de Litchfield et Wilcoxon, s'est inscrite dans un intervalle allant de* 32 *à* 13,5 *cellules, pour l'ensemble des expériences. L'analyse des résultats montre que la* D_{37} *de la courbe de survie des cellules anoxiques (irradiation de l'animal mort à des doses allant de* 480 *à* 3.840 *rad) est environ de* 380 *rad et le chiffre d'extrapolation environ de* 10. *Le taux de l'effet-oxygène a été de* 3. *La comparaison de ces résultats avec ceux obtenus par d'autres chercheurs sur la leucémie des souris, suggère que la* D_{37} *des courbes de survie de cellules d'une tumeur radiosensible ou radiorésistante sont les mêmes, le chiffre d'extrapolation étant probablement différent.*

En délivrant une dose de radiation de 480 *à* 3.840 *rad à de petites tumeurs d'un poids moyen de* 0,4 *g (contenant* 2×10^8 *cellules) chez l'animal vivant non anesthesié la courbe de survie de la tumeur* in vivo *a pu être appréciée. De la forme de ces courbes, on en a*

déduit que 15 *pour cent environ des cellules de ces tumeurs de petite taille ont dû être en état d'anoxie au temps de l'irradiation. La proportion de ces cellules anoxiques, associée à un chiffre d'extrapolation relativement élevé, fournit une explication satisfaisante de la radiorésistance de ce type de tumeur.*

SUMMARY

Using an improved cell dispersion technique, survival curves have been obtained for X-irradiated cells of a transplantable solid rat tumour (rhabdomyosarcoma). Variable doses of irradiated or non-irradiated tumour cells were inoculated into isologous recipients. The non-irradiated (control) ED_{50} *calculated according to the method of Litchfield and Wilcoxon varied from* 32 *cells to* 13·5 *cells for the different experiments.*

The results show that the D_{37} *of the anoxic cell survival curve (irradiation in the dead animal with doses from* 480–3840 *rad) is about* 380 *rad, the extrapolation number N is approximately* 10. *An oxygen enhancement ratio of* 3 *was found. Comparison of these figures with results obtained by others with a murine leukaemia suggests that the* D_{37} *of the survival curves for cells from radiosensitive and radioresistant tumours are similar, the extrapolation number probably being different.*

By delivering doses between 480–3840 *rad to small tumours with an average weight of* 0·4 *g (containing* 2×10^8 *cells) in the living non-anaesthetized animals, the survival curve of the* in vivo *tumour was estimated. From the shape of the various curves it was deduced that* 15 *per cent of the cells of these small tumours has probably been in an anoxic state at the time of irradiation. This proportion of anoxic cells, together with a rather high extrapolation number, provide a satisfactory explanation for the radioresistance of this type of tumour.*

ZUSAMMENFASSUNG

Mittels einer verbesserten Zelldispersions-Technik wurden Überlebungskurven für Zellen eines transplantierbaren soliden Rattentumors (Rhabdomyosarcoma) erhalten. Die bestrahlten oder nichtbestrahlten Tumorzellen wurden in verschiedenen Dosen in isologe Empfänger injiziert.

Die ED_{50} *nicht bestrahlter Zellen (Kontrolle), berechnet nach Litchfield und Wilcoxon, variierte zwischen* 32 *und* 13,5 *Zellen in den verschiedenen Experimenten. Die Ergebnisse zeigen, dasz die* D_{37} *der anoxischen Zellüberlebungs-Kurve (Bestrahlung im toten Tiere mit Dosen von* 480–3840 *rad) ungefähr* 380 *rad beträgt und dasz die Extrapolationszahl N zirka* 10 *ist.*

Eine Sauerstofferhöhungsratio von 3 *wurde gefunden. Eine Vergleichung dieser Zahlen mit Resultaten über Mause Leukämie von anderen Untersuchern suggerieren, dasz die* D_{37} *der Überlebungskurven von Zellen eines strahlungsempfindlichen und eines strahlungsresistenten Tumors sich ähnlich sind, die Extrapolationszahl aber vielliecht verscheiden ist.*

Die Überlebungskurve des in vivo *Tumors wurde bestimmt durch Bestrahlung kleiner Tumoren in den lebenden, nicht narcotisierten Tieren mit einem mittleren Gewicht von* 0,4 *g* (2×10^8 *Zellen). Die Strahlungsdoses variierten von* 480–3840 *rad.*

Aus der Form der verschiedenen Kurven wurde abgeleitet, dasz etwa 15 *Prozent der Zellen dieser kleinen Tumoren zur Zeit der Bestrahlung wahrscheinlich anoxisch waren. Dieser Prozentsatz anoxischer Zellen ergibt, zusammen mit einer ziemlich hohen Extrapolationszahl, eine zureichende Erklärung für die Strahlungsresistenz dieses Tumortyps.*

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The Enhancement of the After Effect of Ionizing Radiation by a Cytotoxic Methylhydrazine Derivative

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INTRODUCTION

EVIDENCE has been published recently for the synergistic effect between the cytotoxic methylhydrazine derivative N-isopropyl-p-(2-methylhydrazino-methyl) benzamide hydrochloride (Natulan®) and ionizing radiation on DNA degradation, the greatest effect being obtained when the compound is added immediately after termination of irradiation [1]. It was demonstrated that this combined action causes more double breaks in the DNA molecule than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent. It had been shown previously that hydrogen peroxide, which is formed [2, 3] and activated [4, 5] during autoxidation of Natulan, is responsible for the DNA degradation by the latter. Interestingly enough no synergistic effect has been obtained when Natulan was added *before* the irradiation. This is probably due to competitive radioprotective reactions of Natulan *during* irradiation resulting from its reducing power, e.g. "repair" of DNA radicals. Similar effects were observed by Butler *et al.* [6, 7] in the case of cysteine which protects DNA when present during irradiation but accelerates the rate at which the after effect occurs when added immediately after irradiation.

In the following, experimental results will be presented which show that the synergism between radiation and Natulan is much more pronounced than the synergism between radiation and hydrogen peroxide. It will further be demonstrated that the synergism between radiation and Natulan decreases with increasing time interval between the end of irradiation and the addition of Natulan, whereas in the case of nitrogen mustard the extent of DNA degradation is not dependent on the time interval between the end of irradiation and the addition of the compound. Finally dose-effect curves are presented which clearly prove the synergism between Natulan and ionizing radiation.

METHODS

Experimental details have been described before [1–4]. The results presented in the following were obtained with sodium deoxyribonucleate (DNA) prepared from calf thymus glands. The average molecular weight of the preparation used was about three millions. A 0·05% solution of DNA in 1/30 molar phosphate buffer was prepared containing 10% sodium chloride to stabilize the double helix structure of the DNA [8]. Sodium pyrophosphate 0·002 moles/l were added to remove iron

ions [9]. For the irradiation experiments a cobalt-60 γ-ray source of 112 c was used [10]. Ten millilitres of the solutions in cylindrical flasks were placed at such a distance from the radiation source that they received the various doses required within 1 hr. Irradiation was carried out at room temperature. After termination of the irradiation the samples were stored at 37°C. The samples to which the compounds were added without preceding irradiation were treated in the same manner. The cytotoxic compounds Natulan(R) [11–13], and the nitrogen mustard methyl-bis(β-chloroethyl)-amine hydrochloride were added in solid form. Hydrogen peroxide was added as 30% aqueous solution (*pro analysi*, Merck Darmstadt). All experiments were carried out in air.

The degradation of the DNA was followed by viscosity measurements and by the determination of sedimentation constants. The viscosities were determined in an Ostwald type viscometer under a sheer stress of about 200–500 sec^{-1} at 37°C. Specific viscosities were calculated according to Staudinger and Heuer [14]. The sedimentation constants of the DNA before and after degradation were determined with a Spinco analytical ultracentrifuge type E at 20°C in the concentration range between 0·02 and 0·05% and extrapolated to the concentration limes zero.

RESULTS

1. *Differences between Natulan and hydrogen peroxide with respect to synergism with ionizing radiation.*

Butler *et al.* [6, 7] have shown that DNA irradiated with X-rays in solution is more susceptible to hydrogen peroxide than unirradiated samples when hydrogen peroxide is added not later than about 20 hr after irradiation. Natulan yields about the equimolar amount of hydrogen peroxide in aqueous solution in the presence of air as a result of its autoxidation [2, 3]. We therefore compared the effect of equimolar amounts of Natulan and of hydrogen peroxide on irradiated DNA solutions. The compounds were added immediately after termination of irradiation. In Fig. 1 the results of these experiments are presented. They show that the synergism between Natulan and irradiation is much more pronounced than between hydrogen peroxide and irradiation. The synergism between irradiation and Natulan can therefore not be explained simply as a result of the hydrogen peroxide formation by the latter compound.

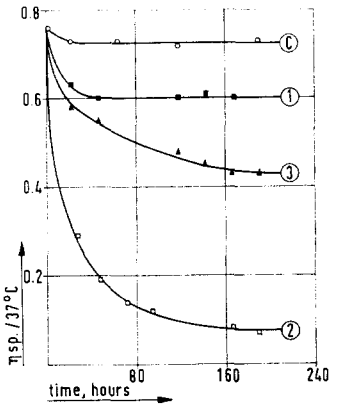

Fig. 1. Change in the specific viscosity of a 0·05% *solution of DNA after the following treatments:*

C. Control (without treatment).

1. Irradiation with 11,000 *rad.*

2. Irradiation with 11,000 *rad and addition of* 0·0005 *moles/l N-isopropyl-p-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan* (R)*),* immediately after *termination of irradiation.*

3. Irradiation with 11,000 *rad and addition of* 0·0005 *moles/l hydrogen peroxide* immediately after *termination of irradiation.*

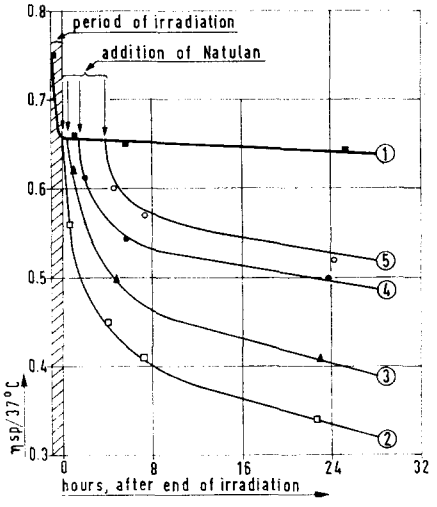

Fig. 2. Change in the specific viscosity of a 0·05% *solution of DNA after irradiation with* 11,000 *rad and addition of* 0·0005 *moles/l N-isopropyl-p-(2-methylhydrazino-methyl)-benzamide hydrochloride (Natulan) at various time intervals.*

1. Irradiated only, no addition of Natulan.

2. Addition of Natulan immediately after termination of irradiation.

3. Addition of Natulan 30 *min after termination of irradiation.*

4. Addition of Natulan 90 *min after termination of irradiation.*

5. Addition of Natulan 235 *min after termination of irradiation.*

2. *Influence of the time interval between irradiation and addition of Natulan on the degradation of DNA.*

Curve 1 of Fig. 2 shows the reduction in viscosity of the DNA solution during 1 hr of irradiation with 11,000 rad which is followed by a further but rather slow decrease of viscosity due to the "after effect" [6, 7, 15–29]. The curves 2 to 5 present the comparatively rapid reduction in viscosity which is induced by the addition of 0·0005 moles/l Natulan(R) to the irradiated solution. The results of curve 2 were obtained with a solution to which Natulan was added immediately after termination of irradiation, whereas in the case of curves 3, 4 and 5 increasing time intervals were inserted between the end of irradiation and the addition of Natulan. These curves demonstrate the very pronounced viscosity decrease which is obtained when Natulan is added to the DNA solution immediately after termination of irradiation, and that the effect of Natulan on the viscosity of irradiated DNA solutions decreases rapidly with increasing intervals between end of irradiation and addition of Natulan. DNA is apparently most susceptible to degradation by Natulan immediately after irradiation. It is assumed that this is due to peroxide products of the DNA formed in the course of irradiation. Most of these unstable products are known to decay within the first few hours after irradiation [6, 19, 30–35].

Figure 3 is a further demonstration of the dependence of the combined effect of Natulan and irradiation on the time interval between irradiation and addition of the compound. Curve 1 shows the specific viscosity 240 hr after the addition of Natulan as a function of the time elapsed between end of irradiation and addition of the compound. This dependence of the reactivity of irradiated DNA solutions on their age is not observed with nitrogen mustard (curve 2). The action of this alkylating type of cytotoxic compound on DNA is apparently not influenced by the unstable products formed during the irradiation of DNA solutions.

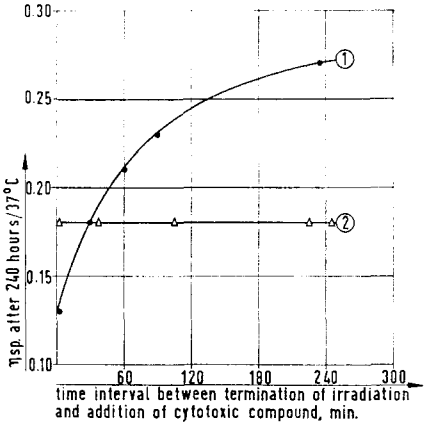

Fig. 3. Specific viscosity of a 0·05% solution of DNA irradiated with 11,000 rad and treated with the cytotoxic compounds Natulan or nitrogen mustard. Influence of the time interval between termination of irradiation and addition of the cytotoxic compounds on the specific viscosity (240 hr after irradiation).

1. 0·0005 *moles/l N-isopropyl-p-(2-methylhydrazinomethyl)benzamide hydrochloride (Natulan(R)).*
2. 0·0005 *moles/l nitrogen mustard [methyl-bis(β-chloroethyl)amine hydrochloride].*

3. *Quantitative differences in the effects of Natulan and of nitrogen mustard on radiation-induced DNA degradation.*

Approximately the same decrease in viscosity of the 0·05% DNA solution is obtained with 0·0005 moles/l Natulan as with 0·0005 moles/l nitrogen mustard 240 hr after the addition of these compounds. However, when these compounds are given to freshly irradiated DNA solutions the additional effect due to irradiation on the viscosity decrease is much smaller in the case of nitrogen mustard than in the case of Natulan. This is made evident by the experimental results given in Table 1.

4. *Synergistic effect between varying doses of Natulan and a fixed radiation dose.*

In Fig. 4 sedimentation constants are presented for DNA degraded by different doses of Natulan with and without preceding irradiation. In the case of the irradiated samples Natulan was added immediately after irradiation. As has been demonstrated in the previous section the greatest effect with respect to viscosity decrease is obtained under these circumstances. After the addition of Natulan, all the samples were stored for 300 hr at 37°C.

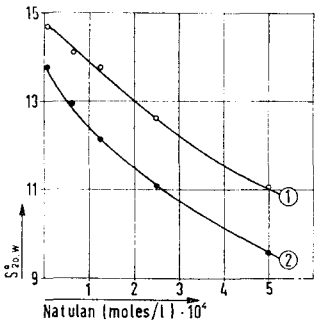

Fig. 4. Sedimentation constants of DNA after degradation with different doses of N-isopropyl-p-(2-methylhydrazinomethyl)benzamide hydrochloride (Natulan (R)) with and without preceding irradiation with 11,000 rad (addition of the compound immediately after termination of irradiation).

1. *Degradation by the compound alone.*
2. *Degradation by the combined action of the compound and ionizing radiation (11,000 rad).*

Table 1. *Specific viscosities of an* 0·05% *DNA solution* 240 *hr after irradiation, after addition of the cytotoxic compounds, and after combination of irradiation and cytotoxic compounds*

Irradiation only (11,000 rad)	Nitrogen mustard only (0·0005 moles/1)	Irradiation +nitrogen mustard	Natulan only (0·0005 moles/1)	Irradiation +Natulan (R)
0·61	0·27	0·21	0·28	0·08

No further decrease of viscosity was observed after this time. The sedimentation constants given in Fig. 4 were obtained by extrapolation to the concentration limes zero.

In Fig. 5 the sedimentation constants of Fig. 4 were converted into "average molar concentrations" of DNA. For this calculation the relationship of Doty *et al.* [36] between average molar weight of degraded DNA and its sedimentation constant was used. Each fracture of the double chain of a DNA molecule yields a new DNA molecule. Therefore the molarity of the solution with respect to DNA increases proportionally to the number of double breaks. It follows that the increase in "average molar concentration" of the DNA is a measure for the number of double breaks that have occurred in DNA molecules. Figure 5 represents the increase of the molarity of the DNA as a function of the concentration of Natulan added to the solution with and without preceding irradiation. From this graph it is seen that the "dose-effect curve" for the combined action of Natulan and irradiation is steeper than the corresponding curve for Natulan alone. The effect of irradiation is thus not just "additive" to the effect of Natulan, in which case two parallel curves should have been obtained. The effect of the two treatments is evidently greater than would be expected from a simple linear superposition.

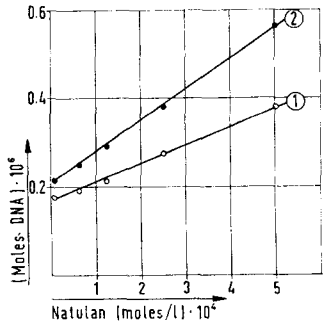

Fig. 5. *Average molar concentration of DNA solutions with respect to DNA after treatment with different doses of N-isopropyl-p-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan(R)) with and without preceding irradiation with* 11,000 *rad (addition of the compound immediately after termination of irradiation.)*

1. *Effect of Natulan(R) alone.*
2. *Effect of the combined action of Natulan(R) and of ionizing radiation* (11,000 *rad*).

5. *Synergistic effect between varying doses of radiation and a fixed dose of Natulan*

In Fig. 6 sedimentation constants are presented for DNA degraded by different doses of radiation with and without addition of a fixed dose of Natulan immediately after irradiation. The samples were stored for 300 hr at 37°C before the analysis was done.

Figure 7 gives the "average molar concentrations" of DNA calculated from the sedimentation constants of Fig. 6 according to the method described in the previous section. Again the curve for the combined action of irradiation and Natulan is steeper than the corresponding curve for irradiation alone, indicating that more DNA molecules are degraded by the combined treatment than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent.

DISCUSSION

The results presented here give further evidence that the combination of ionizing radiation and of Natulan(R) acts in a synergistic and not in an additive way on DNA. A mutual influence of the two treatments on each

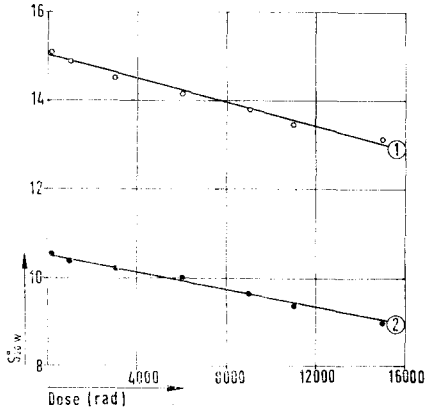

Fig. 6. *Sedimentation constants of DNA after degradation by different doses of radiation with and without addition of N-isopropyl-p-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan(R)) (addition of the compound immediately after termination of irradiation).*

1. *Irradiation alone.*
2. *Combined action of radiation and of* 0·0005 *moles/*1 *Natulan(R).*

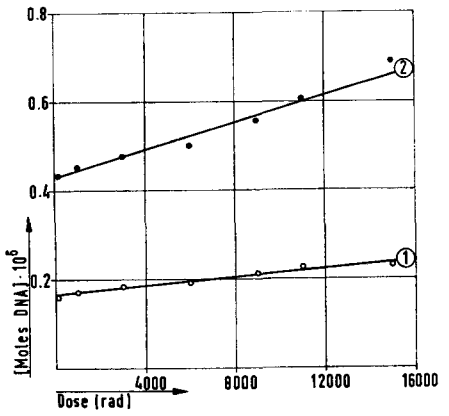

Fig. 7. Average molar concentration of DNA solutions with respect to DNA after degradation by different doses of radiation with and without addition of N-isopropyl-p-(2-methylhydrazinomethyl)benzamide hydrochloride (Natulan(R)) (addition of the compound immediately after termination of irradiation).

1. *Degradation by irradiation alone.*
2. *Degradation by the combined action of radiation and* 0·0005 *moles/l Natulan(R).*

other must be assumed. No synergism was observed when irradiation was combined with either nitrogen mustard or hydrogen peroxide. As the synergistic effect decreases as the time interval between the end of irradiation and the addition of Natulan increases, it is assumed that unstable products formed during irradiation may be responsible for the synergism. Weiss *et al.* [30–32] and Butler *et al.* [6, 18] have shown that organic peroxides are formed during irradiation of DNA solutions which show a marked post-irradiation decay. Butler *et al.* [6, 7] suggests that this decay is even promoted by reducing substances ("reductive activation" [37]). The free radicals formed during this decay may act as initiators [37–39] of the autoxidation [2] of Natulan(R). Earlier experimental evidence [4, 5] supports the view that the autoxidation of Natulan is accelerated by reactions which supply free radicals, such as Fenton chain reactions. Radical scavengers, on the other hand, have a deleterious influence on the autoxidation of Natulan [3]. The higher rate of autoxidation will result in an increase of both the formation [2, 3] and the activation [4, 5] of hydrogen peroxide and thus enhance the degradation of DNA.

It had been claimed before that the action of Natulan on DNA *in vitro* shows marked similarities with the indirect effect of ionizing radiation [16], such as the intermediate formation of hydrogen peroxide [2, 3] and of strongly oxidizing and reducing free radicals [2–5]. The results presented here give further support to this view. They are also of interest in connection with the mechanism of the autoxidation of Natulan for which a chain reaction involving free radicals is assumed [35, 40]. Finally the experimental results may have some bearing on the interpretation of the "after effect" [6, 7, 15–29] of ionizing radiation. It has been claimed by several authors [16, 30–32] that there may be some connection between the "after effect" of ionizing radiation and the post irradiation decay of DNA-hydroperoxides. Our experimental results give further evidence to support this assumption.

RÉSUMÉ

Un dérivé cytotoxique de la méthylhydrazine, le chlorhydrate de N-isopropyl-p-(2-méthylhydrazinométhyl)benzamide (Natulan(R)), dégrade l'ADN en solution par un mécanisme comparable à celui des radiations ionisantes. L'action combinée d'une radiation ionisante et du Natulan(R) provoque des doubles ruptures des chaînes ADN en plus grand nombre que la somme de leurs effets isolés. Cette synergie est démontrée par la relation dose-effet. On obtient l'effet synergique maximum en ajoutant le Natulan(R) sitôt après cessation de l'irradiation. Aucune synergie n'a été observée par combinaison de radiations et d'un composé cytotoxique du type agent alcoylant ("moutarde azotée"). On suppose que l'effet synergique observé avec les radiations et le Natulan(R) est dû aux peroxydes instables, formés durant l'irradiation de l'ADN.

SUMMARY

The cytotoxic methylhydrazine derivative N-isopropyl-p-(2-methylhydrazino-methyl) benzamide hydrochloride (Natulan(R)) degrades DNA in solution by a similar mechanism to ionizing radiation. The combined treatment of DNA with ionizing radiation and with Natulan(R) causes more double breaks of DNA molecules than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent. The synergism between ionizing radiation and Natulan(R) is demonstrated by means of dose-effect curves. To obtain the greatest synergistic effect Natulan(R) has to be added

immediately after termination of irradiation. No synergism can be observed between irradiation and a cytotoxic compound of the alkylating type ("Nitrogen Mustard".) It is suggested that the unstable peroxide products formed during irradiation of DNA are responsible for the synergism between irradiation and Natulan(R).

ZUSAMMENFASSUNG

Die cytotoxische Methylhydrazin-Verbindung N-Isopropyl-p-(2-methylhydrazinomethyl) benzamid-hydrochlorid (Natulan(R)) bewirkt ähnlich wie ionisierende Strahlen einen Abbau von Desoxyribonucleinsäure in vitro. Bei Kombination von Natulan mit ionisierender Strahlung ist der Effekt grösser als die Summe der Einzeleffekte. Der Synergismus zwischen ionisierender Strahlung und Natulan kann anhand von Dosis-Wirkungs-Kurven nachgewiesen werden. Der stärkste Effekt wird erhalten, wenn Natulan(R) unmittelbar nach Beendigung der Bestrahlung zugesetzt wird. Das alkylierende Cytostaticum Methyl-bis-β-chloraethylamin-hydrochlorid ("Nitrogen Mustard") zeigt im erwähnten Modellsystem keinen Synergismus mit ionisierender Strahlung. Der beobachtete Synergismus wird als Folge der Bildung instabiler peroxidischer Produkte während der Bestrahlung erklärt.

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Action du Natulan dans 94 Cas de Tumeurs Solides

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LE CHLORHYDRATE de p-(N'-méthyl-hydrazinométhyl)-N-isopropyl-benzamide ou Natulan* est un dérivé de la méthylhydrazine [1] qui a été utilisé, au cours des premiers essais cliniques, sous le numéro-code Ro 4-6467.

L'expérience clinique de cette substance, dans la maladie de Hodgkin, est déjà assez importante et tous les auteurs s'accordent à lui trouver une efficacité certaine dans cette maladie [2–7]. Un plus petit nombre de résultats favorables ont été enregistrés dans le lymphosarcome, le réticulo-sarcome et la leucémie myéloïde chronique [2–7]. Une étude du groupe coopérateur "leucémies et hématosarcomes" du GECA† a conclu à l'absence d'efficacité du Natulan dans le myélome multiple [8].

Les résultats dans les tumeurs solides sont beaucoup moins nombreux. Au début, d'Alessandri et col. n'avaient observé aucun effet objectif dans une petite série de tumeurs solides [2]. Dans les publications plus récentes de ce groupe, il a été rapporté quelques succès thérapeutiques (parmi 25 cas de tumeurs solides), notamment dans le cancer pulmonaire, dans un cas de sarcome polymorphe et dans un cas de mastocytome [4, 9]. Pernus et col. ont observé un effet objectif dans un cas de carcinome de l'ovaire [10]. Dans 10 cas de tumeurs solides, Gerhartz n'a pas constaté une seule régression objective [11]. Deux améliorations modérées ont été notées par Vicente et col. [12] sur 12 cas traités. Falkson et col. ont traité 39 cas de tumeurs solides; 8 améliorations objectives ont été obtenues, mais seulement chez des malades traités simultanément par le Natulan et par radiothérapie [6]. Todd a traité 6 mélanomes malins et une carcinomatose d'origine inconnue; un cas de mélanome a présenté un effet objectif certain [7]. Brulé et col. rapportent des résultats nettement plus favorables: sur 15 cas de tumeurs solides, traités par le Natulan seul, 5 améliorations objectives (3 cancers pulmonaires, 1 cancer du sein, 1 sarcome fibroblastique) [13]. Ces différentes publications rassemblent un total de 74 cas de tumeurs solides, traités par le Natulan seul, plus un nombre indéterminé de cas de la série de Falkson.

Le présent travail est basé sur 94 cas de tumeurs solides (à l'exclusion des lymphomes malins), traités du 1er juillet 1963 au 30 juin 1965.

MATERIEL ET METHODES

1. *Choix des malades*

Le matériel clinique de cette étude consistait en 94 malades, âgés de 36 à 80 ans, dont 73 étaient du sexe masculin (voir Tableau 1). Tous les malades étaient atteints d'une affection néoplasique maligne. Les lymphomes malins et les leucémies avaient été exclus de cette étude. Le diagnostic a toujours été établi par des examens anatomopathologiques. Tous les malades étaient au-delà des ressources thérapeutiques de la chirurgie et de la radiothérapie et, pour les cancers du sein, de l'hormonothérapie. Vingt-huit malades

*Le Natulan (Trade Mark) nous a été aimablement fourni par les Produits Roche S.A. (Bruxelles).

†Groupe Européen de Chimiothérapie Anticancéreuse.

Tableau 1

Types de tumeurs	Nombre de cas
Epithélioma épidermoïde de la langue	7
Epithélioma épidermoïde de la cavité buccale	4
Adénocarcinome du palais	1
Epithélioma épidermoïde de l'hypopharynx	2
Epithélioma épidermoïde du naso-pharynx	3
Epithélioma épidermoïde de l'amygdale	2
Mélanome malin de l'amygdale	1
Carcinome du sinus maxillaire	1
Epithélioma épidermoïde des fosses nasales	2
Epithélioma épidermoïde du larynx	11
Tumeur mixte de la parotide	1
Epithélioma épidermoïde du poumon	35
Carcinome du poumon	2
Carcinome de l'ovaire	5
Carcinome du sein	7
Epithélioma épidermoïde du col utérin	1
Adénocarcinome du rectum	2
Carcinome à cellules claires du rein	2
Epithélioma épidermoïde de la verge	1
Fibrosarcome du sein	1
Mélanome malin de la peau	1
Rhabdomyosarcome de la fesse	1
Epithélioma épidermoïde d'origine inconnue	1

n'avaient reçu aucun traitement avant le Natulan. Quarante et un malades avaient été traités par la chirurgie, 49 par la radiothérapie et 13 par divers agents chimiothérapiques (ypérite azotée, cyclophosphamide, chlorambucil, 5-fluoro-uracile, mitomycine C, SP-G et SP-I,VER 76). Parmi les cas traités par chimiothérapie, un seul avait répondu favorablement au dernier traitement précédant le Natulan. Il s'agissait d'un cancer pulmonaire traité par l'association de 5 FU et de cyclophosphamide [14].

La mise au point préalable au traitement et la surveillance des malades furent identiques à celles utilisées dans un travail précédent [5].

2. *Mode d'administration du médicament*

Le Natulan a été administré exclusivement par voie buccale chez 59 malades, exclusivement par voie intraveineuse chez 27 malades et par ces deux méthodes chez 7 malades. Un patient a reçu 4 injections intra-artérielles de 100 à 500 mg dans la carotide externe.

La dose quotidienne maximum, par voie buccale, a été de 150 mg dans 2 cas, 200 mg dans 2 cas, 250 mg dans 1 cas, 300 mg dans 56 cas et 400 mg dans 5 cas.

La dose maximum, administrée en une injection intra-veineuse, a été de 250 mg dans 2 cas, 500 mg dans 18 cas, 750 mg dans 6 cas, 1000 mg dans 7 cas, 1250 mg dans 1 cas. Le médicament a été donné en injections intraveineuses rapides, au rythme d'une injection par jour maximum. La majorité des malades a reçu une injection de 500 mg tous les deux jours. Un malade a reçu 7 injections quotidiennes successives de 1000 mg.

La dose totale, par malade, a varié de 250 mg à 30 g (moyenne 8,4 g) et la durée du traitement de 1 à 148 jours (moyenne 34 jours). La dose totale moyenne pour les 80 cas évaluables était de 9,5 g. On s'est toujours efforcé de donner la dose maximum tolérée; le traitement a donc, en principe, été poursuivi jusqu'à l'apparition de signes de toxicité. Il en a été effectivement ainsi dans 58 cas (arrêt pour vomissements intolérables, pour leucopénie en dessous de 3.000 globules blancs, pour thrombocytopénie en dessous de 100.000 plaquettes ou pour diverses autres complications). Le traitement a été interrompu par le décès du malade dans 15 cas. Deux malades étaient encore en traitement au moment de la préparation du manuscrit. Dans les 19 cas restants, le traitement a été interrompu parce que l'état des malades se détériorait rapidement, malgré l'administration d'une dose jugée suffisante (dose totale de 6,5 à 10 g dans 9 cas et de 10,5 à 27 g dans 10 cas). Le Natulan a toujours été utilisé seul. Aucun malade n'a reçu d'autres agents chimiothérapiques, ni de traitement hormonal ou radiologique, pendant toute la durée du traitement au Natulan.

3. *Evaluation du traitement*

Les critères d'évaluation sont les suivants:
Un *effet objectif certain* (+) est défini par une diminution évidente des lésions mesurables, sans aucun signe d'évolution, pendant une durée d'au moins un mois.

Un *effet objectif probable* (±) est défini par:

1. une diminution des lésions mesurables comme ci-dessus, survenant pendant une période inférieure à 1 mois;

2. une diminution évidente des lésions non objectivement mesurables (par exemple, lésions observées par cystoscopie ou par laryngoscopie);

3. une stabilisation de toutes les lésions pendant au moins 6 mois, si ces lésions étaient en évolution avant le traitement.

Nous avons également évalué l'effet du traitement selon la classification de Karnofsky [15]. (cf. Tableau 2).

Tableau 2. Effet thérapeutique du Natulan dans les tumeurs solides

Type de tumeurs	Nbre de cas	Cas évaluables	Effet subjectif seul	Effet objectif probable	Effet objectif certain	Echecs
Tête et cou	35	31	0	2*	3†	26
Poumons	37	31	1	0	3‡	27
Ovaires	5	4	0	0	1§	3
Seins	7	6	0	0	0	6
Divers	10	8	0	0	2‖	6
Totaux	94	80	1	2	9	68

*Catégorie selon Karnofsky [15] : I – A^2, I – A^6

†I – B^{10}, I – A^2, I – A^{2+}

‡I – A^1, I – A^{2+}, I – A^{6+}

§I – A^3

‖I – A^2, I – A^{2+}

RESULTATS

1. *Effet thérapeutique*

Quatorze malades ne sont pas évaluables parce qu'ils ont reçu une dose considérée comme insuffisante ou parce que la période d'observation a été trop courte. La dose totale a été inférieure à 2 g dans 9 cas. Elle était comprise entre 2 et 3,75 g dans 5 cas pour lesquels la période d'observation a été inférieure à 2 semaines.

Une *amélioration subjective*, en l'absence d'un effet objectif, a été notée chez un seul malade.

Un *effet objectif probable* a été observé chez 2 malades. Dans un cas, il s'agissait d'une diminution de volume d'une tumeur non mesurable de la base de la langue. Cette rémission partielle dure depuis 2 mois. Dans l'autre cas, on a observé la cicatrisation d'une ulcération de la base de la langue et la diminution de volume d'une récidive non mesurable à ce niveau. La rémission a duré 6 mois.

Un *effet objectif certain* a été décelé chez 9 malades. La plus longue rémission a duré 10 mois. Il s'agissait d'un épithélioma épidermoïde différencié de la base de la langue qui avait récidivé rapidement après radiothérapie. Toute la partie antérieure du cou était envahie par de volumineuses adénopathies fixées. Toutes les lésions avaient complètement disparu après le traitement au Natulan. Une adénopathie cervicale isolée est réapparue 10 mois plus tard et a augmenté de volume, malgré un nouveau traitement au Ro 4-6467. Ce cas peut être classé comme une rémission apparemment complète.

Les autres cas ont eu des rémissions partielles d'une durée de 1 à 6 mois. Quatre malades sont encore en rémission (2 cas d'épithélioma indifférencié à petites cellules du poumon — Fig. 1 — un cas d'epithélioma glandulaire du voile du palais; un cas d'épithélioma indifférencié d'origine inconnue). Les 4 autres cas ont récidivé dans les trois mois et ils ont tous été résistants à un deuxième traitement au Natulan. Parmi ceux-ci, signalons un cas d'épithélioma glandulaire du côlon, devenu résistant au 5-fluorouracile. L'effet objectif consistait en une diminution de volume des adénopathies inguinales et d'une récidive dans la cicatrice péritonéale, pendant une durée de 2 mois.

L'évolution a progressé malgré le traitement chez 68 malades. L'ensemble des résultats est indiqué au Tableau 2.

2. *Effets sur l'hématopoïèse*

Le Tableau 3 montre l'effet du traitement sur l'hémoglobine, les globules blancs et les plaquettes.

L'anémie (hémoglobine inférieure à 10 g pour 100 ml) est apparue chez 24 malades sur 78 cas évaluables, soit 31 %. Elle a parfois été très sévère, mais elle ne s'est jamais accompagnée de signes évidents d'hémolyse. La bilirubine plasmatique non conjuguée, n'a jamais dépassé les valeurs normales. L'excrétion urinaire d'uribilinogène n'a été augmentée que dans 1 cas. Les réticulocytes ont varié de façon peu systématique. Ils étaient plus fréquemment diminués qu'augmentés. Le fer plasmatique était souvent augmenté, mais cette élévation peut signer l'arrêt de l'érythropoïèse, plutôt que l'hémolyse.

Les leucocytes sont tombés en dessous de 3.000 par mm^3 dans 45 cas sur 77 (58%). Ils sont tombés à moins de 1.500 dans 19 cas. Dans la majorité des cas, la chute des leucocytes

Tableau 3. Toxicité hématologique

	Total des cas traités	Présente	Absente	Non évaluable
Anémie *	94	24	54	16
Leucopénie †	94	45	32	17
Thrombocytopénie ‡	94	35	42	17

*Hémoglobine, moins de 10 g pour cent.

†Leucocytes, moins de 3.000 par mm³.

‡Plaquettes, moins de 100.000 par mm³.

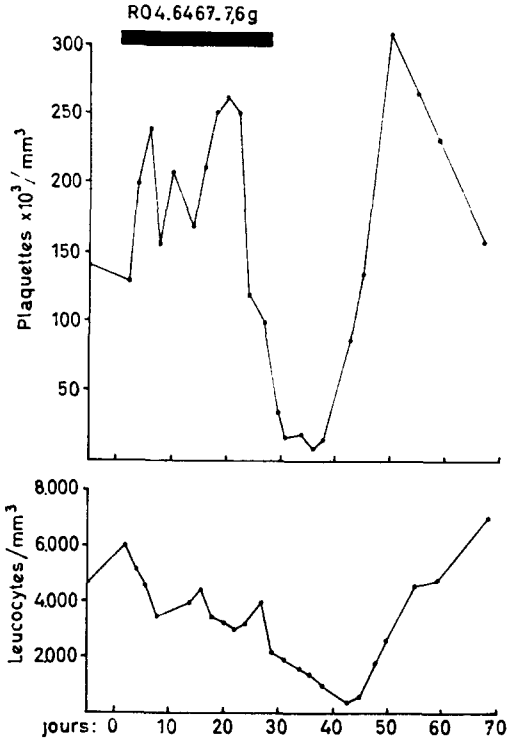

Fig. 2. Effet du Natulan sur les leucocytes et les plaquettes. Cas n° 3.

se poursuivait pendant 2 ou 3 semaines après l'arrêt du traitement (Fig. 2).

Les plaquettes sont tombées en dessous de 100.000/mm³ dans 35 cas sur 77 (45%). Elles sont tombées à des valeurs inférieures à 30.000 dans 13 cas. Un malade a présenté du purpura et un autre malade des hémoptysies, pendant cette période de thrombocytopénie. La diminution des plaquettes est souvent plus brutale que celle des leucocytes, mais le retour à la normale est, lui aussi, plus rapide (Fig. 2).

Cinq cas de cancer du sein, sur 7 traités, ont manifesté une sensibilité extrême du tissu hématopoïétique au traitement. Dans un cas, les leucocytes sont passés de 8.200 à 2.200 par mm³ après une dose totale de 600 mg. Dans 3 cas, les leucocytes sont tombés en dessous de 1.000 par mm³ pour des doses totales de 3.425 mg à 4.950 mg (Fig. 3). Enfin, dans le dernier cas, une dose totale de 2.250 mg en 10 jours a fait tomber les plaquettes de 209.000 à 12.000 par mm³. Dans ce dernier cas, et dans 2 des cas précédents, la moelle osseuse était normale, immédiatement avant le traitement. La moelle n'avait pas été examinée dans les 2 autres cas, mais les examens hématologiques étaient normaux, sans signe d'anémie myélophtisique. Une telle sensibilité n'a été observée chez aucun malade des autres catégories. Dans 3 cas, la leucopénie et la thrombocytopénie ont été irréversibles et ces malades sont morts de septicémie ou d'infection pulmonaire (dose totale : de 7,5 à 10,4 g, en 29 à 37 jours).

3. *Autres signes de toxicité*

La toxicité digestive a été importante; elle n'a été totalement absente que chez 23 malades. Elle a même provoqué l'apparition de vomissements chez 44 malades. Ces vomissements étaient parfois limités à la période initiale du traitement, mais ils étaient persistants et pénibles dans la majorite des cas.

Douze fois, le traitement a été abandonné par le malade, à cause des troubles digestifs. La diarrhée a été présente chez 3 malades. Un malade a présenté un ictère avec altérations des tests hépatiques (élévation de la phosphatase alcaline, des transaminases, des déhydrogénases isocitrique et lactique, de l'aldo-

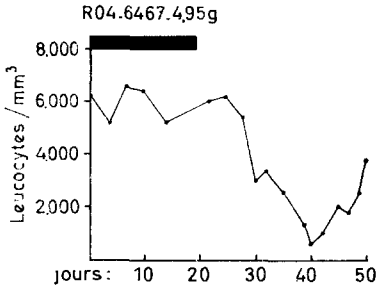

Fig. 3. Effet du Natulan sur les leucocytes dans un cas de carcinome du sein. Cas n° 80.

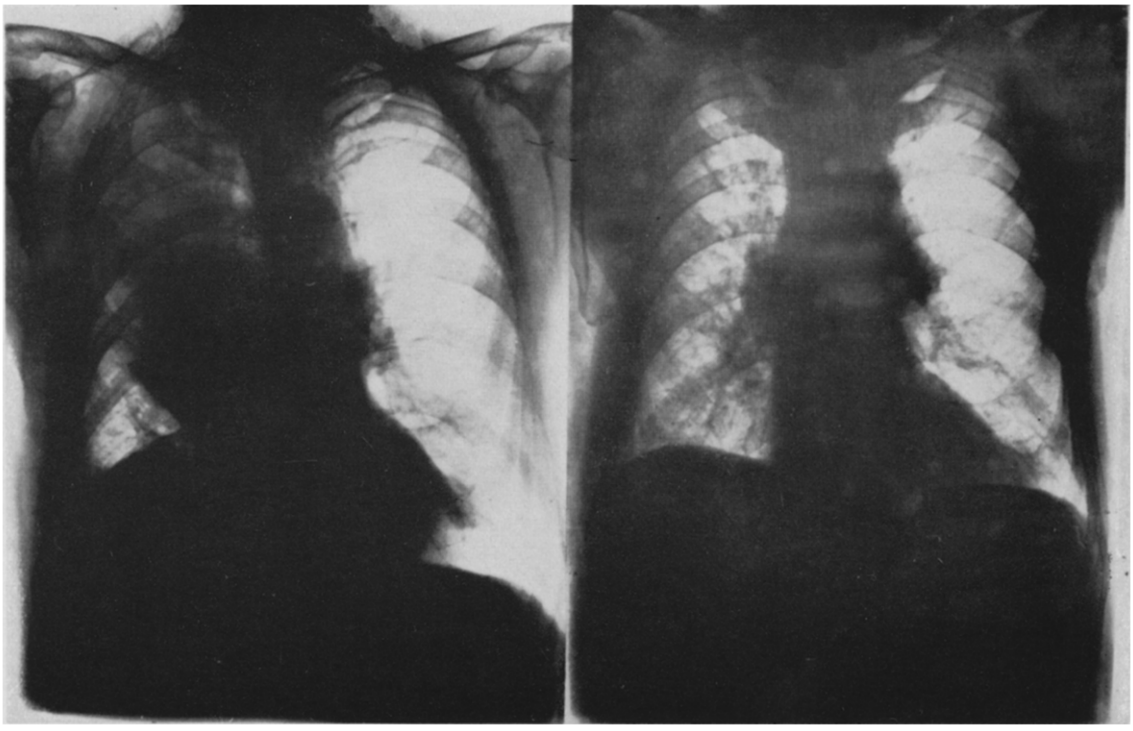

Fig. 1. *Cas n°* 38. *Epithélioma épidermoïde, à petites cellules, du poumon. Dose totale de Natulan :* 21, 45*g*.

(to face p. 54)

lase et de la triosephosphate-isomérase). Ces altérations ont été transitoires. L'existence d'une thrombocytopénie a empêché de faire une biopsie hépatique. L'intolérance à l'alcool (flush syndrome) a été observée chez 9 malades. Une éruption érythémato-papuleuse ou érythémato-squameuse a été observée chez 4 malades.

4. *Décès*

Vingt-cinq malades étaient en vie au moment de la préparation du manuscrit. La durée de la survie, depuis le début du traitement, variait de 2 1/2 à 18 mois (moyenne 8 mois).

Soixante-neuf malades sont morts de 3 à 406 jours après début du traitement (moyenne 101 jours). Dans 66 cas, la cause du décès a pu être rattachée directement à l'évolution du cancer ou à une complication de celui-ci, sans rapport avec une éventuelle toxicité du Natulan. Nous avons cité plus haut les 3 décès par infection, au cours d'une période de leucopénie sévère, secondaire au traitement.

DISCUSSION

Notre expérience avec le Natulan, dans les tumeurs solides, confirme que cette substance est capable de provoquer des rémissions objectives dans un petit nombre de cas de cancers généralisés. La plus longue rémission a duré 10 mois. Les effets objectifs observés dans un cas de carcinome de l'ovaire et dans trois cas de cancer pulmonaire auraient vraisemblablement pu être obtenus par l'administration d'agents alkylants.

Le pourcentage de rémissions dans le cancer du poumon est plutôt faible (3 cas sur 31 malades évaluables) et probablement inférieur à ce qu'on pourrait attendre de certains autres agents chimiothérapiques. La survie moyenne de nos cas, à partir du 1er jour de traitement, est de 88 jours, ce qui correspond exactement à la survie médiane des malades de Wolf recevant un placebo et est inférieure d'un mois à la survie des cas traités par ypérite azotée [16]. Chez 2 de nos 3 malades où un effet objectif certain a été observé, il s'agissait d'épithélioma indifférencié à petites cellules (oat cells). L'histologie était de ce type chez 16 malades. Nos résultats dans le cancer bronchique semblent inférieurs à ceux de Brulé [13]. Cet auteur a observé 3 rémissions objectives sur 5 cas traités. La dose moyenne utilisée par nous (et la toxicité observée) est cependant plus importante. Deux malades de Brulé, ayant présenté une rémission, ont reçu le Natulan par voie intraveineuse. Dans notre série, les 3 rémissions ont été observées à la suite d'un traitement oral et cinq de nos cas ont reçu le Natulan par voie intraveineuse, sans succès.

Cinq cas de tumeurs de la tête et du cou (4 cavités buccales et 1 larynx) ont été améliorés objectivement par le traitement au Natulan. Nous avions observé 7 effets objectifs sur 23 patients de la même catégorie et évalués selon les mêmes critères, traités par la mitomycine C [17]. Sur 6 malades de ce groupe, résistants au Natulan, 2 ont présenté une amélioration objective certaine, à la suite d'un traitement ultérieur à la mitomycine C. Il n'y a donc pas de résistance croisée entre ces deux produits. L'hétérogénéité des tumeurs de la tête et du cou, la faiblesse des effectifs de chaque localisation, les conditions différentes d'observation, ne permettent pas de comparer l'efficacité du Natulan à celle d'autres agents capables de provoquer des régressions dans les tumeurs de ce type, comme par exemple, la vinblastine [18] et le Méthotrexate [19]. Tout ce que l'on peut dire, c'est que l'efficacité de ces traitements doit varier approximativement entre 10 et 30%. Nous ne pouvons pas non plus affirmer que certaines localisations soient plus sensibles que d'autres à la chimiothérapie, bien que, dans notre série traitée au Natulan et dans celle de Smart, traitée à la vinblastine, les rémissions soient plus fréquentes dans les néoplasmes de la cavité orale que dans les épithéliomas du larynx.

Une rémission a été obtenue dans un cas de carcinome du côlon devenu résistant au 5-fluoro-uracile.

Les effets toxiques constatés correspondent à ceux observés dans les lymphomes malins et les leucémies [5]. La fréquence de la leucopénie est pratiquement identique dans les 2 séries (58% dans les tumeurs solides, 60% dans les lymphomes). La thrombocytopénie est un peu plus fréquente dans les lymphomes et les leucémies (73% contre 45%). Les troubles digestifs sont également fréquents dans les deux groupes (73 et 64%). Un ictère n'a été observé que dans un seul cas de la série actuelle et on peut se demander s'il s'agit d'une réaction toxique au dérivé de la méthyl-hydrazine. La mortalité attribuable au traitement est faible: 2% dans les lymphomes et 3,2% dans les tumeurs solides.

CONCLUSIONS

Le chlorhydrate de p-(N'méthyl-hydrazino-méthyl)-N-isopropyl-benzamide ou Natulan est actif dans environ 10% des cas de tumeurs

solides. Quelques observations permettent de penser qu'il n'y a pas de résistance croisée entre ce produit et le 5-fluoro-uracile et la mitomycine C. Les études dans la maladie de Hodgkin avaient déja montré qu'il n'existait pas de résistance croisée avec la vinblastine et les agents alkylants. La toxicité, avec les doses et le mode d'administration utilisés, est relativement faible. Les effets observés dans les épithéliomas de la tête et du cou incitent à poursuivre l'investigation de ce produit dans ce type d'affections néoplasiques et à comparer son efficacité à celle de la mitomycine C, de la vinblastine et du Méthotrexate.

RESUME

Quatre-vingt-quatorze cas de tumeurs solides ont été traités par le Natulan. Un effet objectif certain a été observé dans 3 *cas d'épithélioma de la tête et du cou,* 3 *cas de cancer du poumon,* 1 *cas de carcinome de l'ovaire,* 1 *cas de carcinome du côlon et* 1 *épithélioma épidermoïde d'origine inconnue. Deux effets objectifs probables ont été observés en outre dans des épithéliomas de la tête et du cou.*

La toxicité hématologique a été modérée et toujours réversible, sauf chez 3 *patients qui sont morts d'infection avec leucopénie. La toxicité digestive a été le principal inconvénient du traitement.*

SUMMARY

Ninety-four patients with solid tumours have been treated by Natulan. A definite objective response has been noted in 3 *cases of head and neck carcinoma,* 3 *cases of lung cancer,* 1 *case of ovary carcinoma,* 1 *case of colon carcinoma and* 1 *case of epidermoid carcinoma of unknown origin. Two questionable objective responses have also been observed in head and neck carcinomas.*

The hematological toxicity has been moderate and always reversible except in 3 *patients who died of infection with leukopenia. The main drawback of the treatment consisted in gastrointestinal toxicity.*

ZUSAMMENFASSUNG

Vierundneunzig Tumorfälle sind mittels Natulan behandelt worden. Gesicherte, objektive Resultate sind in 3 *Fällen von Epithelioma des Kopfes, in* 1 *Fall von Ovarialkarzinom, in* 1 *Fall von Dickdarmkarzinom, in* 1 *Fall eines epidermoiden Epitheliomas unbekannten Ursprungs beobachtet worden. Ausserdem kam es in* 2 *Fällen von Epitheliomata des Kopfes und des Halses zu wahrscheinlichen objektiven Besserungen.*

Die haematologische Toxizität war mässig und immer reversibel, abgesehen von 3 *Patienten, welche an Infektionen begleitet von Leukopenie starben. Die toxische Wirkung auf den Verdauungstraktus war die Hauptschwierigkeit im Laufe der Behandlung.*

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Klinische und lymphographische Verlaufskontrollen bei Lymphogranulomatose und Retothelsarkom unter Behandlung mit Natulan®

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NATULAN®, zunächst unter der Prüfbezeichnung Ro 4-6467 bekannt, ist ein uns seit April 1963 zur experimentellen und klinischen Prüfung zur Verfügung stehendes Zytostatikum aus der Klasse der Methylhydrazin-Derivate, deren tumorhemmende Wirkung erstmals von Bollag und Grunberg [1] beschrieben wurde. Chemisch handelt es sich um das p-(N'-methylhydrazinomethyl) - N - isopropyl - benzamide - hydrochlorid.

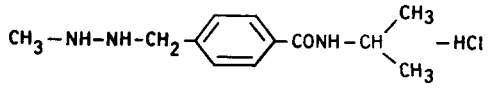


Abb. 1. *p-(N'methylhydrazinomethyl)-N-isopropylbenzamid-hydrochlorid = Natulan.*

Nachdem der onkolytische Effekt des Natulans auf eine Reihe transplantabler Tiertumoren bereits bekannt war, stellten wir selbst eine deutliche Wachstumshemmung bzw. vollständige Regression in Abhängigkeit von Tumorgröße und Dosis beim Jensen-Sarkom der Ratte fest [2].

Ferner konnten wir in tierexperimentellen Untersuchungen zeigen, daß sich Natulan in seiner biochemischen Wirkung deutlich von einer großen Zahl anderer zytostatisch wirksamer Verbindungen unterscheidet. Während alkylierende Substanzen wie Äthyleniminochinone und Chloroethylamine (Lost-Derivate) aber auch nichtalkylierende Verbindungen wie Vincaleukoblastinsulfat, einen Abfall des Gehaltes an NAD (Nikotinamid-adenindinukleotid, früher DPN) und einen konsekutiven Anstau der phosphorylierten Intermediarprodukte der Glykolyse Triosephosphat und Fruktosediphosphat im Tumorgewebe und im Gewebe schnellproliferierender Organe hervorrufen [3], konnte im Jensen-Sarkom nach Natulan-Gaben ein solcher Effekt nicht nachgewiesen werden [2]. Weiterhin blieb im mit Natulan behandelten Tumor die durch andere Zytostatika aus den obengenannten Gruppen in der Warburg-Apparatur nachgewiesene Hemmung der anaeroben Glykolyse und Senkung der Gewebsatmung aus [3].

Inzwischen weisen einige Untersuchungen auf eine Beeinflussung des Nukleinsäurestoffwechsels durch Natulan hin. Nachdem Berneis *et al.* [4] *in vitro* einen Viskositätsabfall von Desoxyribonukleinsäure unter Natulan-Einwirkung feststellen konnten, zeigten Fölsch, Drews und Grunze [5] eine Verringerung des Einbaus von markiertem Glyzin unter Natulan bei gleichzeitig verminderter Thymidin-Aufnahme, was für eine Hemmung sowohl der DNS als auch der Proteinsynthese spricht.

Ob die Wirkung auf die DNS durch einen Alkylierungsvorgang zustande kommt [6], ist noch nicht sicher bewiesen; in eigenen, noch nicht veröffentlichten Versuchen konnten wir vorerst keinen Anhalt für alkylierende Eigenschaften von Natulan finden.

Nachdem auf Grund der tierexperimentellen Befunde das Natulan eine gewisse Sonderstellung unter den Zytostatika einnahm, interessierte vor allem seine klinische Wirksamkeit. Erste Ergebnisse am Menschen wurden von D'Alessandri, *et al.* [7], Martz *et al.* [8], Brunner *et al.* [9], Mathé *et al.* 1963 [10], Mathé *et al.* 1964 [11], Gerhartz [12] und Kenis [13] mitgeteilt. Wir berichten im Folgenden über unsere Erfahrungen mit Natulan in der Behandlung der Lymphogranulomatose und des Retothelsarkoms. Die Resultate, die bei anderen malignen Neoplasien gewonnen wurden, werden anderenorts publiziert werden.

KRANKENGUT

In der Zeit von April 1963 bis November 1964 haben wir in unserer Klinik 18 Patienten mit histologisch gesicherter Lymphogranulomatose und 3 Patienten mit histologisch gesichertem Retothelsarkom mit Natulan behandelt. Es handelt sich um 12 Männer und 9 Frauen.

1. *Lymphogranulomatosen*

Zur Behandlung kamen 10 männliche Patienten zwischen 21 und 60 Jahren mit einem Durchschnittsalter von 37 Jahren. Das Alter der Frauen variierte zwischen 23 und 43 Jahren mit einem Durchschnitt bei 32 Jahren.

Die *Dauer der Erkrankung*, gerechnet vom Zeitpunkt der histologischen Sicherung an bis zum Behandlungsbeginn mit Natulan, lag zwischen 3 Wochen und 144 Monaten, im Mittel bei 36 Monaten.

Eine besondere Patientenauswahl für den Einsatz des Natulans wurde nicht getroffen; so lag ein recht gemischtes Krankengut vor. Dies zeigt sich einmal bei Betrachtung der schon angeführten unterschiedlichen Dauer der Erkrankungen, zum anderen aber auch an Hand der unterschiedlichen *Vorbehandlungen*. 4 Patienten waren nicht vorbehandelt, bei 14 Patienten waren früher eine oder mehrere spezifische Behandlungen durchgeführt worden. Diese bestanden in 3 Fällen ausschließlich in Röntgen-Tiefentherapie, in 2 Fällen ausschließlich in medikamentöser Therapie mit Zytostatika. Die restlichen 9 Kranken waren schon sowohl röntgenbestrahlt als auch zytostatisch behandelt worden.

In 3 Fällen bestand eine *Resistenz* gegenüber Zyklophosphamid, 2 davon waren zusätzlich auch gegen Vincaleukoblastin resistent. Bei allen anderen vorbehandelten Patienten hatte die letzte Behandlung zu einer mehr oder weniger vollständigen Remission geführt.

Unterschiedlich war auch die Zahl der stattgehabten *Rezidive nach früheren Behandlungen*. Neben den 4 frischen, unbehandelten Erkrankungen lagen in 8 Fällen 1. Rezidive, in 6 Fällen 2. bis 4. Rezidive vor.

Nach dem Grad der Ausbreitung der Erkrankung wurden 2 *Stadien* unterschieden, das multinoduläre und das Generalisationsstadium, wobei unter einer Generalisation das gleichzeitige Vorhandensein von Lymphomen in Lymphknotenregionen sowohl cranial als auch caudal der Zwerchfellgrenze bzw. das zusätzliche Auftreten von Leber-, Milz- oder Knochenherden zum multinodulären Befall verstanden wurde.

2. *Retothelsarkome*

Unter diesem Krankheitsbild kamen nur 2 Männer und eine Frau im Alter von 56, 61 und 70 Jahren zur Behandlung mit Natulan.

In allen Fällen handelte es sich um generalisierte Erkrankungen. Die Krankheitsdauer seit histologischer Diagnosestellung lag bei allen 3 Patienten unter 4 Wochen. Bei einem Kranken bestanden allerdings seit bereits 13 Monaten massive, bis kleinkindskopfgroße Lymphome, ohne daß therapeutisch eingeschritten worden wäre.

Nur ein Patient, nämlich derjenige mit der längsten klinischen Symptomatik, war vorbehandelt und zwar zytostatisch mit Vincristinsulfat, gegenüber dem er primär resistent war.

BEHANDLUNGSMODUS

Ursprünglich stand uns Natulan nur in Ampullen zu je 250 mg zur Verfügung. 6 Patienten wurden mit i.v.-Injektionen behandelt, von denen 2, nachdem Natulan auch in Drageeform vorlag, oral weiterbehandelt wurden. 14 Patienten wurde Natulan asschließlich oral appliziert.

Die *Behandlungsdauer* lag zwischen 9 und 140 Tagen.

Wir gingen nach 2 *Behandlungsplänen* vor: Zu Beginn der Erprobung des Präparates wurde die Behandlung mit einer Natulan-Menge von 100 mg täglich begonnen und die tägliche Dosis innerhalb von 3–4 Tagen auf 250–500 mg erhöht. Diese Dosis wurde während längstens 3 Wochen beibehalten und dann die Natulan-Behandlung beendet, einerlei ob eine Remission eingetreten war oder nicht. Es wurde dann entweder eine Röntgen-Tiefentherapie oder die Behandlung mit einem der

bereits erprobten Zytostatika angeschlossen. Zu dieser Gruppe gehören 9 Patienten.

Bei den später zur Behandlung gekommenen Patienten wurde nach initialem Einschleichen eine Tagesdosis von 300 mg verabreicht und diese Dosis auch über eine etwaige Remission hinaus im Sinne einer zytostatischen Rezidivprophylaxe beibehalten. Aus noch zu erörternden Gründen wurde in der letzten Zeit die Dauerbehandlungsdosis auf 150 mg/die festgelegt.

Bei allen Patienten wurde mit der Therapie unter stationärer Kontrolle begonnen. In 7 Fällen wurde die Dauerbehandlung ambulant weitergeführt unter Blutbildkontrolle des Hausarztes und eingehenden klinischen Kontrolluntersuchungen in Abständen von längstens 6 Wochen.

Die verabreichten *Gesamtdosen* schwankten zwischen 2 und 30 g Natulan, dies entsprach Werten zwischen 40 und 430 mg/kg Körpergewicht.

Als *Zusatzbehandlung* wurden nur in 3 Fällen von Lymphogranulomatosen und in einem Fall eines Retothelsarkoms Corticoide verabreicht. Die übrige medikamentose Therapie beschrankte sich auf symptomatische Maßnahmen, besonders zur Linderung des Brechriezes. Zu diesem Zweck wurde anfanglich Benadon® i.v., später Haloperidol® in Tropfenform oder als i.v.-Injektion gegeben. Wahrend der Dauertherapie wurde in einigen Fallen Durabolin® bzw. Decadurabolin® verordnet.

BEURTEILUNG

Für die klinische Beurteilung des Therapieerfolges wurde, neben dem klinischen Untersuchungsbefund, den Laboratoriumsuntersuchungen und den üblichen röntgenologischen Untersuchungsmethoden die Lymphangioadenographie [14] eingesetzt, die sich zur Erfassung von Großen- und Strukturveränderungen der Lymphknoten im Bereich des Retroperitonealraums und des Beckens anbot. Die Beurteilung wurde so durchgeführt, daß zwischen "sehr guten," "guten" und "mäßigen" Remissionen und "stationärem oder progredientem" Verlauf unterschieden wurde. Wir werteten nur solche klinischen Besserungen, die mindestens einen Monat anhielten.

In den vorliegenden Fällen von Krankheiten des lymphatischen bzw. lymphoretikulären Zellsystems waren für die Beurteilung objektivierte Größenänderungen palpabler oder röntgenologisch nachgewiesener Lymphome bzw. Knochenherde maßgebend. Insbesondere bei der Beurteilung lymphographischer Befunde wurden vergleichende Messungen einzelner vergrößerter und pathologisch strukturierter Lymphknoten vor, während und nach Abschluß der Natulan-Behandlung durchgeführt, um eine möglichst genaue Objektivierung des Therapieerfolgs zu erreichen (Tab. I).

Die Messungen wurden bei den einzelnen Patienten immer an den gleichen Lymphknoten vorgenommen. Differenzen von 1,0 mm liegen im Fehlerbereich der Meßgenauigkeit. Zu beachten ist ferner, daß die Lymphknoten während der Lymphangioadenographie eine geringe Größenzunahme durch Kontrastmittelüberangebot erfahren. Die ursprüngliche Lymphknotengröße ist dann aber nach 8–14 Tagen meist wieder erreicht.

Alleinige Besserungen unspezifischer Symptome wie Blutsenkungsgeschwindigkeit, Elektrophorese, Blutbild und subjektiver Symptome galten noch nicht als Remisionen. Im einzelnen bedeutete:

"sehr gute Remission":	Normalisierung aller pathologischen Befunde einschließlich der subjektiven Beschwerden und der blutchemischen Veränderungen,
"gute Remission":	Rückbildung des überwiegenden Teils der krankheitsspezifischen Befunde,
"mäßige Remission":	Rückbildung einzelner krankheitsspezifischer Befunde,
"keine Remission"	stationärer Befund oder progredienter Verlauf.

THERAPEUTISCHE ERGEBNISSE

1. *Bei der Lymphogranulomatose*

In Tab. I sind die lymphographischen Verlaufsbeobachtungen von Größenänderungen iliakaler und paraaortaler Lymphknoten unter Natulantherapie zusammengestellt.

In den Fällen I–V handelt es sich um Patienten, bei denen die Lymphographie das typische Bild eines lymphogranulomatösen Befalls der Lymphknoten mit Größenzunahme und spezifischen Strukturveränderungen zeigte und bei denen ein besonders eindrucksvoller Rückgang der Lymphknotenschwellungen zu beobachten war wie aus den Meßergebnissen ersichtlich ist. Die Abb. 2a, b, c demonstrieren das am Fall V.

Tabelle 1. *Lymphographische Verlaufsbeobachtungen von Größenänderungen iliakaler und paraaortaler Lymphknoten vor, während und nach Natulan-Behandlung. Die Messungen erfolgten immer an den gleichen Lymphknoten.*

Fall	Name	Diagn:	Untersuchungsdatum E: Erstuntersuchung K: Kontrolluntersuchung	Nach Gesamtdosis Natulan absolute Menge in g	Nach Gesamtdosis Natulan mg/kg Körpergewicht	Lymphknotengröβer (mm) (Breiten- und Längsdurchmesser) Iliacal Rechts	Iliacal Links	Paraaortal Rechts	Paraaortal Links
I	S.A.	Lgr.	E: 16.4.63	—	—	12–27	8–23	8–21	8–33
			K: 3.5.63	8,5	145	11–28	7–22	8–15	7–30
			K: 20.5.63	8,5	145	11–25	7–20	7–14	7–26
			K: 19.6.63	8,5	145	11–24	6–20	8–13	
II	D.H.	Lgr.	E: 3.1.64	—	—	12–18	11–12	14–29	11–18
			K: 7.4.64	4,4	47	10–18	10–12	13–25	9–13
III	J.A.	Lgr.	E: 10.7.63	—	—	8–32	6–16	8–22	10–20
			K: 20.8.63	7,3	111	8–28	5–15	6–22	10–20
			K: 2.1.64	7,3	111	8–27	5–12		10–19
IV	B.M.	Lgr.	E: 22.5.64	—	—	13–34	12–25		
			K: 18.6.64	8,1	133	8–25	7–21		
			K: 16.7.64	16,5	257	6–24	4–14		
			K: 11.9.64	28,5	468	6–17	5–11		
V	W.R.	Lgr.	E: 3.1.64	—	—	8–18	9–17	7– 9	16–22
			K: 27.1.64	—	—	9–17	8–16	6– 9	17–23
			K: 26.2.64	—	—	7–15	9–16	5– 7	20–22
			K: 10.4.64	—	—			8–10	26–27
			K: 5.6.64	14,7	228	7–13	6–10	6–7	16–17
			K: 21.7.64	27,8	431	7–14	3–7		7–13
VI	H.F.	Lympho-retikuläres Sarkom	E: 15.5.64	—	—	27–49	12–29		15–24
			K: 8.9.64	28,8	436	24–43	9–23		

Bei allen in Tab. 1 angegebenen Fällen von Lymphogranulomatose trat daneben unter Natulan-Behandlung eine mindestens "gute" Remission der übrigen befallenen Lymphknoten, etwa im Mediastinum oder in den cervicalen, supraclaviculären oder axillären Lymphknotenregionen ein.

An Hand von Tab. 2 wird ein Gesamtüberblick über die erzielten therapeutischen Ergebnisse bei der Behandlung der Lymphogranulomatose mit Natulan gegeben.

Dabei ist den Autoren klar, daß die Prozentangaben bei der geringen Zahl von Fallen keinen repräsentativen Charakter besitzen.

Da die Remissionen in den meisten Fällen bereits in den ersten 2–3 Wochen nach Behandlungsbeginn eintraten, konnten Fälle mit Kurzzeitbehandlung und anschließendem Therapiewechsel und Dauerbehandlungsfälle bezüglich der *Remissionshäaufigkeit* und des *Remissiongrades* zusammen beurteilt werden. Dies geht auch aus Tab. 1 hervor: In den Fällen I, II und III wurden Kurzzeitbehandlungen, in den Fällen IV und V Langzeitbehandlungen mit Natulan durchgeführt. In allen Fällen trat die wesentliche therapeutische Wirkung relativ frühzeitig ein.

Betrachtet man das Gesamtkrankengut (Tab. 2), so tritt bei 14 von 18 Lymphogranulomatose-Kranken verschiedener Stadien eine mehr oder minder vollständige Remission ein, das sind 78%. 10 Patienten davon, das sind 56%, zeigen eine mindestens "gute" Remission, d.h. Rückbildung des überwiegenden Teils der krankheitsspezifischen Symptome.

Unterteilt man das Krankengut in entweder radiologisch oder spezifisch chemotherapeutisch vorbehandelte Patienten und solche ohne spezifische *Vorbehandlung* so ergibt sich erwartungsgemäß ein besserer Behandlungserfolg bei den nicht vorbehandelten, frischen Erkrankungen. Bei allen 4 Patienten der letzteren Kategorie war eine "gute" Remission zu erzielen, während die Angehörigen der vorbehandelten Gruppe nur in 42% der Fälle mindestens "gute" Behandlungsergebnisse aufwiesen.

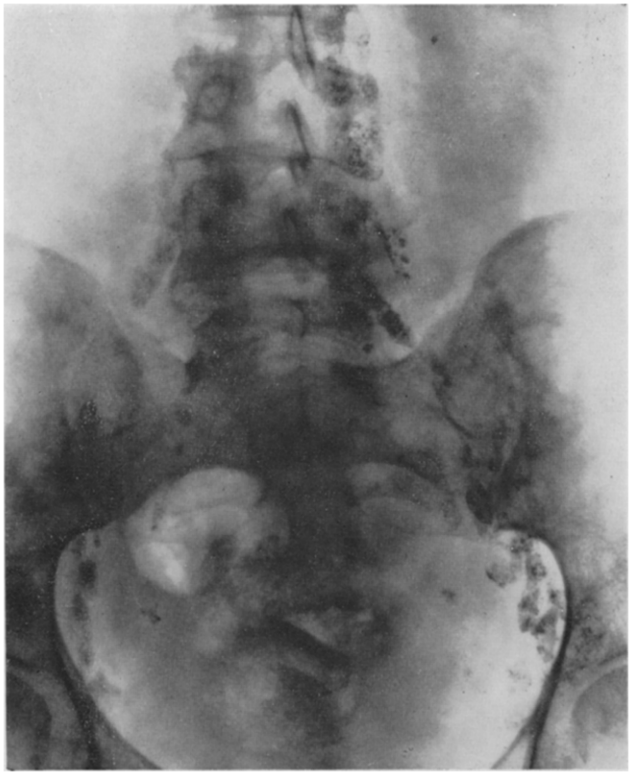
Dagegen zeigt ein Vergleich zwischen Patienten in verschiedenen *Stadien* der Erkrankung keine großen Unterschiede zwischen multinodulärem Befall und bereits eingetretener Generalisation bezüglich der Remissionen. Eine mehr oder weniger vollständige Remission

Abb. 2. Patientin W.R.: Maligne Lymphogranulomatose.

a. *Erstuntersuchung: Deutlich vergrößerte iliakale und paraaortale Lymphknoten links mit Strukturabweichungen, wie man sie bei M. Hodgkin findet. Deutliche Strukturabweichungen auch in normal großen iliakalen und paraaortalen Lymphknoten rechts.*

b. *3 Monate später: Größenzunahme der iliakalen und paraaortalen Lymphknoten links als Ausdruck eines Rezidivs.*

(to face p. 62)



c. *Weitere 2 Monate später: Nach Natulan-Behandlung mit insgesamt 14,7 g Natulan = 228 mg/kg Körpergewicht deutliche Größenabnahme der paraaortalen und iliakalen Lymphknoten links.*

Abb. 3. Patient H.F.: Lymphoretikuläres Sarkom.

a. Erstuntersuchung: Deutlich vergrößerte iliakale Lymphknoten beiderseits mit pathologischer Speicherstruktur.

b. Kontrolle 3 Monate später: Nach Natulan-Behandlung mit insgesamt 28,8 g Natulan = 436 mg/kg Körpergewicht deutliche Größenabnahme der iliakalen Lymphknoten.

Tabelle 2. Behandlungsergebnisse bei Lymphogranulomatose

			Therapieerfolg			
		Fallzahl	+++	++	+	φ
(a) *Multinoduläre Form:* Alleinige Therapie mit Natulan	vorbehandelt	5	1	2	1	1
	nicht vorbehandelt	2	—	2	—	—
(b) *Generalisierte Form:* Alleinige Therapie mit Natulan	vorbehandelt	6	—	3	1	2
	nicht vorbehandelt	2	—	2	—	—
Natulan + Corticoide bzw. Rö.	vorbehandelt	3	—	—	2	1
Total: Alleinige Therapie mit Natulan Natulan + Corticoide		18	1	9	4	4

+++ sehr gute Remission = Normalisierung aller Symptome (klin.u.chem.)
++ gute Remission = Rückbildung des überwiegenden Teils der Symptome.
+ mäßige Remission = Rückbildung nur einzelner Symptome.
φ stationärer oder progredienter Verlauf.

konnte in der ersten Gruppe in 86%, in der zweiten in 73% objektiviert werden.

Der Einfluß einer *zusätzlichen Behandlung mit Corticoiden* konnte nicht beurteilt werden. Von insgesamt 18 Patienten wurde nur in 3 Fällen eine solche zusätzliche Behandlung durchgeführt. In zwei Fällen davon kam es zu einer "mäßigen" Remission, in einem Fall war ein progredienter Krankheitsverlauf zu verzeichnen. In allen 3 Fallen handelt es sich um bereits generalisierte und mehrfach spezifisch vorbehandelte Patienten, bei denen wegen schlechten Allgemeinzustandes die Corticoid-Behandlung indiziert war. Diese Fälle stellen somit eine negative Auslese dar.

Neben der Remissionshäufigkeit und dem Remissionsgrad interessiert natürlich die *Remissionsdauer* besonders. Leider sind darüber noch keine genauen Angaben zu machen, insbesondere da der Beobachtungszeitraum noch relativ kurz ist. Zudem fallen alle diejenigen Fälle, bei denen nach vorher festgelegter Behandlungsdauer mit Natulan ein Therapiewechsel durchgeführt wurde — und das sind gerade diejenigen mit der längsten Beobachtungszeit — für die Beurteilung der Remissionsdauer weg. So können nur diejenigen 5 Patienten für die Beurteilung herangezogen werden, bei denen im Anschluß an eine "gute" bis "sehr gute" Remission die Natulan-Therapie als Dauerbehandlung fortgeführt wurde. 4 dieser 5 Patienten sind z.Z. 16 Wochen, 19 Wochen, 21 Wochen und 30 Wochen nach nachgewiesenem Remissionseintritt noch rezidivfrei. Dagegen trat bei einer vielfach und different vorbehandelten Patientin (Fall V in Tab. 1) nach gutem Rückgang der Lymphome bereits 8 Wochen nach Beginn der Remissions und unter Dauerbehandlung mit Natulan ein lokales Rezidiv auf.

Nicht tabellarisch angeführt, weil für die Beurteilung des Therapieerfolgs nicht ausschlaggebend, wurden die unter Behandlung mit Natulan erzielten Besserungen unspezifischer Laboratoriumsbefunde wie Blutsenkungsgeschwindigkeit und Serumkupfer/Serumeisen-Relation. Fast ohne Ausnahme traten Besserungen unspezifischer Krankheitssymptome nur in Verbindung mit objektiven Besserungen krankheitsspezifischer Befunde auf. Das gleiche gilt für die klinisch so bedeutungsvollen subjektiven Besserungen wie Entfieberung sowie Wegfall von Juckreiz, Alkoholschmerz, starker Neigung zum Schwitzen und Besserung von Müdigkeit und Schlappheit.

2. *Beim Retothelsarkom*

Wie Tab. 3 zeigt, können wir nur über 3 Fälle berichten, die mit Natulan behandelt wurden.

In Tab. 1 ist als Fall VI ein Patient mit einem histologisch gesicherten lymphoretikulären Sarkom angeführt, bei dem die Rückbildung der pathologisch vergrößerten und strukturierten Beckenlymphknoten zahlenmäßig registriert ist. Die Abb. 3a, b zeigen die zugehörigen Lymphangioadenogramme. Gleichzeitig kam es zur fast vollständigen Rückbildung vergrößerter peripherer Lymphknoten in mehreren Regionen sowie zu einer neurologisch objektivierten Besserung einer bestehenden Glossopharyngicusparese.

Tabelle 3. Behandlungsergebnisse bei Retothelsarkom

		Therapieerfolg				
		Fallzahl	+++	++	+	φ
Alleinige Therapie mit Natulan	nicht vorbehandelt	2	—	1	—	1
Natulan + Corticoide	vorbehandelt	1	—	—	1	—
Total: Alleinige Therapie mit Natulan Natulan + Corticoide		3	—	1	1	1

+++ sehr gute Remission = Normalisierung aller Symptome (klin.u.chem.)
++ gute Remission = Rückbildung des überwiegenden Teils der Symptome.
+ mäßige Remission = Rückbildung nur einzelner Symptome.
φ stationärer oder progredienter Verlauf.

In den beiden anderen Fällen von Retothelsarkomen unter Natulan-Dauerbehandlung konnte in einem Fall nur eine "mäßige" Remission durch objektive Größenabnahme der Lymphome einer Region bei stationärem Befund der übrigen befallenen Lymphknotenregionen erzielt werden, im anderen Fall war das Krankheitsbild durch vierwöchige Behandlung mit Natulan nicht zu beeinflussen und zeigte anhaltende Progredienz.

NEBENWIRKUNGEN

1. *Verträglichkeit*

An erster Stelle der Nebenwirkungen stehen Appetitlosigkeit, Übelkeit und *Erbrechen*. Tab. 4 zeigt, daß Erbrechen in fast der Hälfte aller Fälle auftrat und immerhin in 3 Fällen zum endgültigen Absetzen der Natulan-Behandlung zwang. Der Brechreiz trat bevorzugt während der ersten Behandlungstage auf, um später meist vollständig zu verschwinden. Er war von der Einzeldosis relativ unabhangig. Auch sahen wir in dieser Beziehung zwischen intravenoser und oraler Verabreichung keinen Unterschied. Während i.v.-Injektionen von Benadon® die Brechneigung kaum beeinflußten, war mit Haloperidol®, i.v. oder oral meist eine deutliche Besserung zu erzielen.

Demgegenüber spielten die anderen beobachteten Unvertraglichkeitserscheinungen nur eine untergeordnete Rolle. In zwei Fallen sahen wir einen massiven, aber reversiblen *Haarausfall*. In einem Fall wurde über *polyneuritische Beschwerden* in Form von Paraesthesien und neuralgischen Schmerzen, in einem weiteren über Drehschwindel und Kollapsneigung geklagt.

Bei einem Patienten mit Lymphogranulomatose entwickelte sich unter Dauerbehandlung nach einer Gesamtmenge von 29,4 g Natulan = 482 mg/kg Körpergewicht (Fall VI) bei gleichzeitig bestehender Leukopenie von 700/mm³ und Thrombozytopenie von 48 500/mm³ ein *generalisierter Herpes zoster*. Für seine Entstehung dürfte die Abwehrschwache wesentlich mitverantwortlich sein, die nicht nur durch die Grundkrankheit, sondern auch durch die durch Natulan induzierte Granulozytopenie und Immuno-Suppression [15, 16] beding tist.

Ferner ist das Auftreten einer akuten exogenen Psychose mit optischen Halluzinationen zu erwähnen, die nach Absetzen des Präparates rasch abklang.

Tabelle 4. Nebenwirkungen unter Natulan-Therapie bei 21 Patienten

		Unterbrechung oder Absetzen der Natulan-Therapie
Symptome	Fallzahl	(Fallzahl)
Erbrechen	10	3
Massiver Haarausfall	2	—
Toxische Hautreaktion	2	2
Herpes zoster generalisatus	1	1
Kreislaufstörungen	1	—
Polyneuritis	1	—
Akute exogene Psychose	1	1
Leukopenie <2000	6	5
Thrombozytopenie <50 000	4	5

Bei 2 Patienten hatte sich unter Natulan-Behandlung eine toxische, wahrscheinlich allergische Hautreaktion in Form eines makulopapulosen, stark juckenden Exanthems entwickelt, das ebenfalls nach Absetzen von Natulan rasch wieder verschwand. Da beide Falle in kurzem zeitlichen Abstand zueinander und nach Behandlung aus der gleichen Charge auftraten, geht das Exanthem moglicherweise auf eine Verunreinigung des Praparates zurück.

2. *Auswirkungen auf das hämatopoetische System*

Wie unter allen zytostatisch wirksamen Substanzen traten auch unter Natulan Depressionen der Knochenmarksfunktion in Form von *Granulozytopenien* und *Thrombozytopenien* auf. Diese kamen im allgemeinen erst relativ spät zur Ausbildung. In den Fällen von Kurzzeitbehandlung mit anschließendem Therapiewechsel waren die Hämozytopenien nie so ausgeprägt, daß sie zu einer Änderung des Therapieplans Anlaß gegeben hätten. Unter Dauerbehandlung mit 300 mg Natulan täglich kam es meist erst nach 10–12-wöchiger Anwendung, also nach Gesamtmengen von über 20 g, zu Knockenmarksdepressionen.

In 6 Fällen (29%) sank der Leukozytenwert im peripheren Blut unter 2000/mm³, so daß Natulan vorübergehend abgesetzt werden mußte. Im Fall IV der Kasuistik bringen wir, wie schon erwähnt, die Ausbildung des generalisierten Herpes zoster mit der Granulozytopenie und der hemmenden Wirkung des Natulans auf die Antikörperbildung in Zusammenhang. Sonst sahen wir klinisch keine Folgen.

Auch Thrombozytopenien zwangen in 4 Fällen (19%), in denen der Thrombozytenspiegel im peripheren Blut unter 50 000/mm³ herabsank, zu vorübergehender Unterbrechung der Therapie; jedoch haben wir in keinem Fall eine hämorrhagische Diathese beobachtet. Thrombozytopenien und Granulozytopenien traten etwa gleichzeitig miteinander auf.

Eine Beeinträchtigung des *erythropoetischen Systems*, erkenntlich an einer Abnahme der Retikulozytenzahl im peripheren Blut, konnte nicht festgestellt werden. Da von Methylhydrazinen bekannt ist, daß sie eine hämolysierende Wirkung besitzen [17], war eher mit einem kompensatorischen Retikulozytenanstieg im Blut zu rechnen. Zwar konnten wir in einigen Fällen Retikulozytenanstiege bis 60% und entsprechende Anstiege des reduzierten Harnfarbwertes F_0 [18] bis zu 4 als mögliche Folge einer geringgradigen Hamolyse registrieren, jedoch war das rote Blutbild kaum beeinflußt und der Vorgang klinisch bedeutungslos. Heinzsche Innenkorper wurden nie gesehen.

DISKUSSION

Mit Natulan, das sich in seiner chemischen Konstitution von allen bisher gebräuchlichen Zytostatika unterscheidet, konnten in 78% aller bei uns zur Behandlung gekommenen *malignen Lymphogranulomatosen* Remissionen unterschiedlichen Grades erzielt werden.

Erste klinische Untersuchungen mit Natulan waren seit 1962 von D'Alessandri *et al.* [7] durchgeführt worden. In einer ersten Mitteilung wurde von 14 mit Natulan behandelten Lymphogranulomatosen berichtet, von denen 10 einen guten Behandlungserfolg, 4 nur mäßige Remissionen zeigten. Inzwischen sind vom gleichen Arbeitskreis weitere Ergebnisse veröffentlicht worden [8, 9]. In einer Zusammenfassung berichtet Martz [19] von 25 bisher behandelten Lymphogranulomatosen in verschiedenen Stadien, die alle gut auf Natulan ansprachen, indem bei ihnen ein Rückgang aller nachgewiesenen Lymphome auf mindestens 50% der ursprünglichen Größe, bzw. eine meßbare Größenabnahme der vergrößerten Leber oder Milz beobachtet wurde.

Diesem besten Behandlungsergebnis stehen andere gegenüber, die einen gewissen Prozentsatz gegenüber Natulan weitgehend oder vollständig resistenter Fälle von Lymphogranulomatosen aufweisen. Mathé *et al.* [10, 11] erzielten in 76%, Gerhartz [12] in 57% und Kenis [13] in 65% der mit Natulan behandelten Lymphogranulomatosen mehr oder weniger vollstandige Remissionen. Diese Behandlungsergebnisse decken sich in etwa mit den unsrigen.

Diese Erfolgsquote entspricht etwa dem therapeutischen Index anderer bisher in der Therapie der Lymphogranulomatose gebräuchlicher Zytostatika wie Zyklophosphamid [20, 21], oder Vincaleukoblastin [22], wenn der Beurteilung vergleichbare Kriterien zugrundegelegt wurden.

Natürlich ist auch unser Krankengut für eine endgültige Beurteilung zu klein. Es ist das Verdienst der Herstellerfirma von Natulan, der Deutschen Hoffman — La Roche, eine Gruppe von 12 großen deutschen Kliniken zur gleichzeitigen Erprobung, gemeinsamem Erfahrungsaustausch und zu einer Gemeinschaftspubli-

kation veranlaßt zu haben, wobei sich an Hand eines 10-fachen Krankenmaterials, in dem auch unsere Patienten enthalten sind, eine fast gleiche therapeutische Erfogsquote wie bei unserer kleinen Patientenzahl zeigt (im Druck inzwischen erschienen [23]).

Unsere Untersuchungen zeigen, daß Natulan den wirksamsten bekannten Zytostakika an die Seite zu stellen ist. Mit diesen vergleichbar ist auch die Zahl der gegenüber Natulan primär resistenten Patienten von 22%. Eigene Untersuchungen mit Vincaleukoblastin hatten eine primäre Resistenz von 20% aller Fälle ergeben [22].

Was die therapeutische Beeinflußbarkeit der *Retothelsarkome* betrifft, so kann an Hand unserer 3 behandelten Fälle nur gesagt werden, daß in einem Teil der Fälle Remissionen erzielt werden können. Auch die bisher in der Literatur beschriebenen Fälle reichen nicht für eine Beurteilung aus. Mathé [11] berichtet von 11 Patienten mit Retothelsarkomen, von denen 7 unter Natulan-Behandlung Remissionen aufwiesen, Kenis [13] von einem erfolgreich behandelten Fall. Die oben erwähnte Gemeinschaftspublikation wird hier näheren Autschluß bringen inzwischen erschienen [23].

Natulan führt auch nach vorheriger Behandlung mit anderen Zytostatika zu Remissionen. Es konnte von uns gezeigt werden, daß zwischen Natulan und Zyklophosphamid, Vincaleukoblastin und Vincristin keine Kreuzresistenzen bestehen, so daß auch bei Resistenz gegenüber diesen Präparaten eine Anwendung von Natulan noch Aussicht auf Erfolg hat.

Eine günstige Eigenschaft von Natulan ist sein rascher Wirkungseintritt zu einem Zeitpunkt, zu dem bei der erforderlichen Dosierung noch mit keinen hämatologischen Nebenerscheinungen gerechnet werden muß. Subjektive und objektive Remissionszeichen waren in der Regel bereits nach einer Gesamtdosis von 2 g Natulan, die im allgemeinen in der zweiten Behandlungswoche erreicht wird, erkennbar. Höhere Einzeldosen als 300 mg/die schienen nur den Nachteil einer schlechteren Verträglichkeit zu haben, für den Eintritt der Remission aber nicht entscheidend zu sein.

Günstig ist auch die perorale Applikationsmöglichkeit, die Natulan für die ambulante Rezidiv-Dauerprophylaxe geeignet macht.

Nebenwirkungen müssen auch von Natulan in Kauf genommen werden. Im Vordergrund der Unverträglichkeitserscheinungen steht das Erbrechen, das bei uns in 48% aller behandelten Fälle auftrat. Martz [19] gibt Erbrechen in ungefähr 50%, Gerhartz [12] bei 64% aller mit Natulan behandelten Patienten an. Nach unseren Erfahrungen nötigt das Erbrechen aber bei einschleichender Dosierung und Gaben von Antiemetika im allgemeinen nicht zum Absetzen der Therapie.

Weit ernster zu beurteilen sind die Granulozyto-und Thrombozytopenien, die in der Regel aber erst nach Eintritt der Remission während der Dauerbehandlung zur Rezidivprophylaxe schwerere Formen annehmen können. Wir sahen immerhin in 28% aller behandelten Fälle Leukopenien unter 2000/mm³ und in 19% Thrombozytopenien unter 50 000/mm³. Gerhartz [12] fand Leukopenien unter 2000/mm³ nur bei 12%, Kenis [13] dagegen solche unter 3000/mm³ bei 57%, Thrombozytopenien unter 100 000/mm³ sogar bei 68% aller Fälle.

Bei einer täglichen oralen Dosis von 300 mg Natulan traten bei uns schwerere Depressionen der Knochenmarksfunktion meist erst nach 10–12 wöchiger Behandlung entsprechend Gesamtdosen von ca. 20 g Natulan auf. Ein regelmäßig früheres Auftreten der Thrombozytopenien vor den Granulozytopenien, wie es von Martz [19] beschrieben wurde, konnten wir nicht bestätigen. Bei täglichen oralen Dosen von nicht mehr als 150 mg, wöchentlicher Leukozytenkontrolle und Thrombozytenkontrollen in längstens 4-wöchigen Abständen, scheint die Dauerprophylaxe aber ohne stärkere Gefährdung des Patienten durchführbar.

Ausschlaggebend dafür, ob sich Natulan in der Therapie der Lymphogranulomatosen und Retothelsarkome behaupten kann, wird aber die zu erzielende Remissionsdauer sein, über die noch keine allgemeingültigen Aussagen gemacht werden können wegen der Kürze der Beobachtungszeit. Unser längstes beobachtetes rezidivfreies Intervall beträgt bei den Lymphogranulomatosen 30 Wochen, bei den Retothelsarkomen 24 Wochen, ohne daß zum Zeitpunkt der Registrierung Anzeichen für ein Rezidiv bestanden. Die von Martz und Mit. [7, 8, 9, 19] erzielten rezidivfreien Intervalle liegen zwischen 5 und 25 Wochen. Länger andauernde Remissionen konnten bisher nicht beobachtet werden. Auch bei Mathé und Mit. [10, 11] sind die Remissionsdauern sehr unterschiedlich und schwanken zwischen 8 und 28 Wochen. In Übereinstimmung mit diesen Autoren, die Rezidive 5 und 8 Wochern nach Remissionseintritt sahen, konnten wir unter Dauerbehandlung mit Natulan 8 und 12 Wochen nach erfolgter Remission Rezidive beobachten, was für die Möglichkeit einer sich

relativ rasch entwickelnden sekundären Resistenz spricht. Längere Beobachtungszeiten und größeres Krankengut werden darüber näheren Aufschluß bringen.

Anerkennungen—Der Firma Deutsche Hoffman—La Roche in Grenzach danken wir für die freundliche Überlassung der benötigten Mengen von Natulan.

RÉSUMÉ

Le Natulan est un nouveau cytostatique du groupe des dérivés de la méthylhydrazine. Il a été essayé chez 18 *malades atteints de lymphogranulomatose maligne et chez* 3 *patients atteints de réticulosarcome. Dans* 78% *des cas de lymphogranulomatose maligne, l'on a obtenu des rémissions plus ou moins complètes, ce qui range le Natulan parmi les cytostatiques les plus efficaces. Des rémissions ont également été observées dans deux cas de réticulosarcome.*

Les propriétés les plus favorables du Natulan sont le début rapide de l'effet thérapeutique, la possibilité de l'administration perorale et l'absence de résistance croisée vis-à-vis d'autres cytostatiques. Les auteurs insistent sur la possibilité de la prophylaxie des récidives par un traitement cytostatique chez des malades ambulants au moyen du Natulan.

Les effets secondaires, parmi lesquels il faut noter une tendance aux nausées et la possibilité de granulopénie et de thrombopénie, pouvant se déclarer relativement tardivement au cours des traitements prolongés, sont discutés.

SUMMARY

Natulan is a new cytostatic of the group of methylhydrazine derivatives. It has been tried out on 18 *patients with Hodgkin's disease and in* 3 *patients with reticulum cell sarcoma. In* 78% *of Hodgkin's disease cases, more or less complete remissions were obtained which puts Natulan among the most effective cytostatics. Remissions have also been noted in* 2 *cases of reticulum cell sarcoma.*

The most favourable properties of Natulan are the rapid therapeutic effect, the possibility of peroral administration and the absence of cross-resistance with other cytostatics. The authors insist on the possibility of prophylaxis of the recurrences by a cytostatic treatment of out-patients with Natulan.

Side effects, among which a tendency to nausea and the possibility of granulopenia and thrombopenia which can appear late in prolonged treatments, are discussed.

ZUSAMMENFASSUNG

Natulan, ein neues Zytostatikum aus der Gruppe der Methylhydrazin-Derivate, wird bei 18 *Patienten mit Lymphogranulomatosen und* 3 *Patienten mit Retothelsarkomen erprobt. Bei den Lymphogranulomatosen werden in* 78% *der Fälle mehr oder weniger vollständige Remissionen erzielt, was Natulan gleichrangig neben die bisher wirkungsvollsten Zytostatika stellt. Auch bei* 2 *Patienten mit Retothelsarkomen tritt eine Remission ein.*

Als besonders günstige Eingeschaften des Natulans werden rascher Wirkungseintritt, perorale Verabreichungsmöglichkeit und fehlende Kreuzresistenz zu anderen Zytostatika herausgestellt. Die Möglichkeit einer ambulanten zytostatischen Rezidivprophylaxe mit Natulan wird betont.

Die Nebenwirkungen der Natulantherapie, unter denen Neigung zum Erbrechen und eine unter Dauertherapie relativ spät einsetzende Granulozyto- und Thrombozytopenie hervorstechen, werden besprochen.

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A Preliminary Clinical Evaluation of the Therapeutic Effects of Hydroxyurea in Patients with Myelogenous Leukaemia

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As FAR back as in 1928, Rosenthal *et al.* [1] reported the presence of myeloid and erythroid changes induced by hydroxyurea (HU) in the bone-marrow of experimental animals.

After more than 30 years, a revived interest in hydroxylamine derivatives was shown as a result of the report of STEARNS *et al.* [2] on the therapeutic effects obtained with HU in some experimental leukaemias. Because of the limited toxicity of this compound in animal trials, the drug was soon tested for its possible antileukaemic and antitumour effects in man.

The trials so far carried out have shown that HU is inactive or shows only marginal activity in most types of solid tumours [3–5], including those of the lymphoid and reticulo-endothelial system [6–8]. In acute and chronic leukaemias, the only significant therapeutic responses were observed in cases of chronic granulocytic leukaemia [8–9].

In view of the favourable results obtained in the latter condition and of the scarcity of newly synthesized antileukaemic agents, we set out to assess the activity of HU and now report the preliminary results in a group of patients with myelogenous leukaemia.

METHODS AND MATERIAL

Hydroxyurea is a colourless, crystalline compound, readily soluble in water. Its chemical structure is: $NH_2–\overset{\overset{O}{\|}}{C}–NH–OH$.

The drug was supplied in the form† of capsules of 500 mg each for oral use.

On the basis of the results reported by Krakoff *et al.* [9], who obtained satisfactory results in chronic granulocytic leukaemia with initial doses of 40 mg/kg and maintenance doses of 20 mg/kg, treatment in our cases was started with doses varying from 1–3 g, depending on the patient's weight and the level of the leucocyte count. Maintenance doses varied from 0·5–1·5 g.

The cases included in the present trial consisted of one case of subacute myeloblastic-promyelocytic leukaemia, twelve cases of chronic granulocytic leukaemia in the chronic phase and five in blastic crisis.

Routine haematological investigations were carried out in all cases. In three cases of chronic granulocytic leukaemia, leucocytic alkaline phosphatase was assessed with the method described by Hayhoe and Quaglino [10] before and during treatment.

In all cases the leucocyte count was above the normal range and splenomegaly was present. Six cases of chronic granulocytic leukaemia had previously been treated with Myleran and/or X-rays, while the remaining were untreated .

The objective criteria for evaluating the therapeutic response to HU were based on the decrease of the leucocyte count, the reduction in the size of the spleen, a rise in the red cell count and in the haemoglobin level, and on the improvement of the patient's general condition.

*Scholar of the Lady Tata Memorial Trust.

†Simes Ltd. Milan.

RESULTS

Haematological findings

(*a*) *Peripheral blood.* In all cases of chronic granulocytic leukaemia in the chronic phase of the disease, HU in variable doses from 2–3 g caused, within 10–15 days from starting therapy, a decrease in the level of the leucocyte count to near normal or normal levels. With the doses used in the present trial, decreases below 2,000 WBC per mm^3 were never observed. Following the reduction in the numbers of circulating leucocytes, also the differential count tended to become normal.

HU did not cause any depressive effect on the level of the red cell count, but on the other hand it did not appear to improve substantially the anaemia of those patients, in which the red cell count was initially low.

The platelet level was not significantly altered by treatment with HU, except in one case, in which the platelet count dropped to 10,000 per mm^3 following treatment with HU for 2 months. This case, however, had reacted in a similar way when treated previously with Myleran.

In the one case of subacute myeloblastic-promyelocytic leukaemia and in the five cases of chronic granulocytic leukaemia in blastic crisis, HU often succeeded in reducing the initial high leucocyte level, but the decrease was due mainly to the disappearance of the more mature cells of the granulocytic series, the primitive nucleoated cells persisting practically unmodified.

In the three cases in which leucocyte alkaline phosphatase was evaluated, a slight increase over the low scores obtained initially (0–5) was observed in two cases, when the leucocyte count had reverted to normal as a result of treatment with HU. The scores obtained were 15 in one case and 52 in the other (normal range 14–100). In the third case the low enzyme levels were not influenced by HU.

(b) *Bone-marrow aspirations* carried out in some patients during treatment with HU always yielded highly cellular specimens. However even in those cases in which treatment was continued for fairly prolonged periods (10 months), the M:E ratio remained between 4: 1 and 10: 1 and did not differ substantially from the ratio present before starting treatment.

In a proportion of our cases, increased numbers of promyelocytes, some of which showed prominent nucleoli and nuclear abnormalities, were present in the bone-marrow specimens obtained in the course of treatment with HU. Occasionally, following prolonged periods of therapy with HU, slight megaloblastic changes and a moderate degree of macrocytosis were observed in the red cell series, but the phenomenon was never conspicuous.

Clinical Data

A marked reduction in the size of the spleen occurred in all cases treated with HU. In seven cases, during the chronic stage after treatment for one month with the compound, the spleen was no longer palpable. The general conditions of all the patients during the chronic phase appeared to improve with an increase in weight and appetite.

With regard to the patients in blastic crisis and to the case of subacute myeloblastic-promyelocytic leukaemia, no effect was observed on the general conditions of the patients. Two of the cases, subsequently treated with H6-mercaptopurine (6MP), are, at the moment of writing, in complete clinical and haematological remission. Four of the patients instead died soon after treatment with HU had been discontinued and replaced with 6 MP.

HU did not cause in the series of cases studied and at the doses used, any unpleasant side-effects. None of the patients complained of gastro-intestinal symptoms or of any other disturbance.

Representative Case Reports

1. Male, 46 years old; (Fig. 1). Admitted to hospital on the 6th July 1964 and diagnosed as having chronic granulocytic leukaemia. The spleen was palpable 4 fingers below the costal margin and the liver 1 finger. The WBC count was 264,000 per mm^3 with a differential as follows: Myeloblasts 4%, Promyelocytes 35%, Myelocytes 11%, Metamyelocytes 13%, Normoblasts C 1% and Normoblasts B 2%, Neutrophil polymorphs 31%, Basophils 3%. On the 10th of July treatment was started with 3 g of HU daily. Seven days later the WBC count dropped to 25,000 and the dosage was accordingly reduced to 2 g daily. Three days later, since the WBC count had further decreased to 7,400 cells per mm^3 the dosage was cut to 1·5 g *per die*. On the 1st of August the patient was discharged from hospital, with a WBC count of 5,200 leucocytes per mm^3 and a normal differential and on a maintenance dose of 1·5 g daily. After seventeen days, due to unforeseen circumstances, the patient remained without treatment for about 2 weeks. When a routine haematological examination was performed, it was found that the WBC count had risen to 80,000 cells per mm^3 with numerous granulocyte precursors in the peripheral blood.

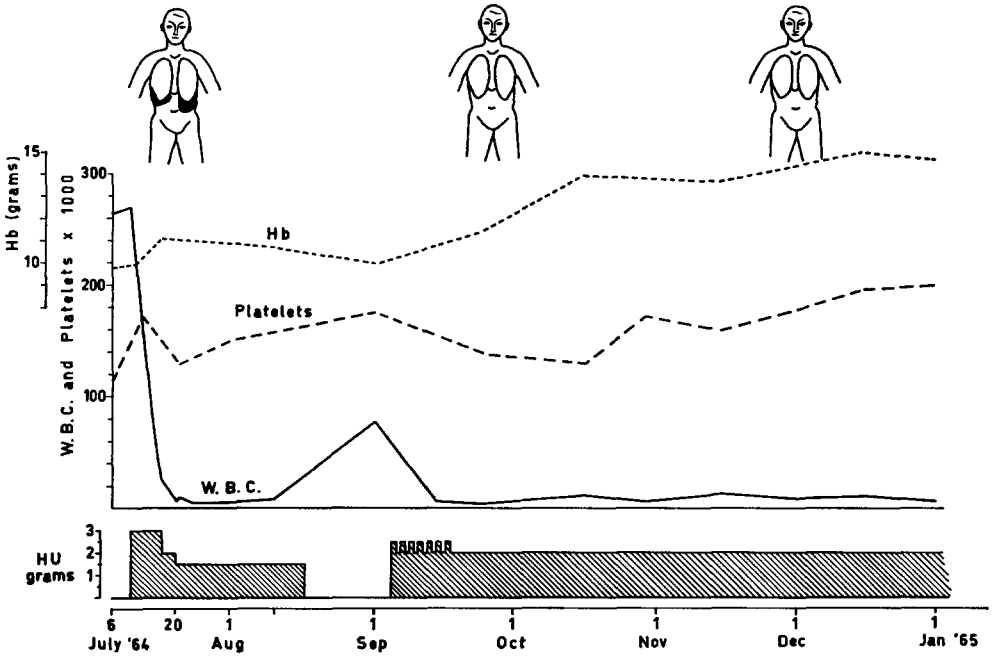

Fig. 1. *Chronic granulocytic leukaemia.*

Treatment was restarted on the 4th September with 4–5 capsules given on alternate days. Ten days later, the WBC count had reverted back to normal limits (6,000 per mm³). Since then the patient has been in good clinical and haematological remission on a maintenance dose of 2 g daily, therapeutic regime which the patient is continuing at the time of writing.

2. Male 36 years old (Fig. 2). Admitted to hospital on the 9th of June 1964 with a diagnosis of subacute myeloblastic-promyelocytic leukaemia. The general condition of the patient was poor, the haemoglobin was 8 g per cent, the WBC count 230,000 per mm³, with a differential as follows: Myeloblasts 10%, promyelocytes 19%, Myelocytes 19%, Metamyelocytes 12%, Normoblasts C 1%, Neutrophil polymorphs 46%, Eosinophils 1%, Basophils 1%, Lymphocytes 1%. On the same day treatment with HU was started with an initial dose of 3 g daily. The count progressively decreased to 98,000 leucocytes per mm³ on the 12th of June and to 39,000 leucocytes per mm³ on the 15th of June. In view of the sharp drop in the WBC count, the dosage of HU had been reduced to 1·5 g daily on the 12th of June. It should be pointed out, however, that although the WBC count had decreased considerably as a result of treatment with HU, the percentage of blast cells had actually risen. On the 20th of June the WBC count increased to 45,000 cells per mm³ and the dosage of HU was stepped up to 3 g daily. However, in spite of this therapeutic measure, the percentage of blast cells continued to rise steadily. It was therefore decided to discontinue treatment with HU and to change over to 6 MP.

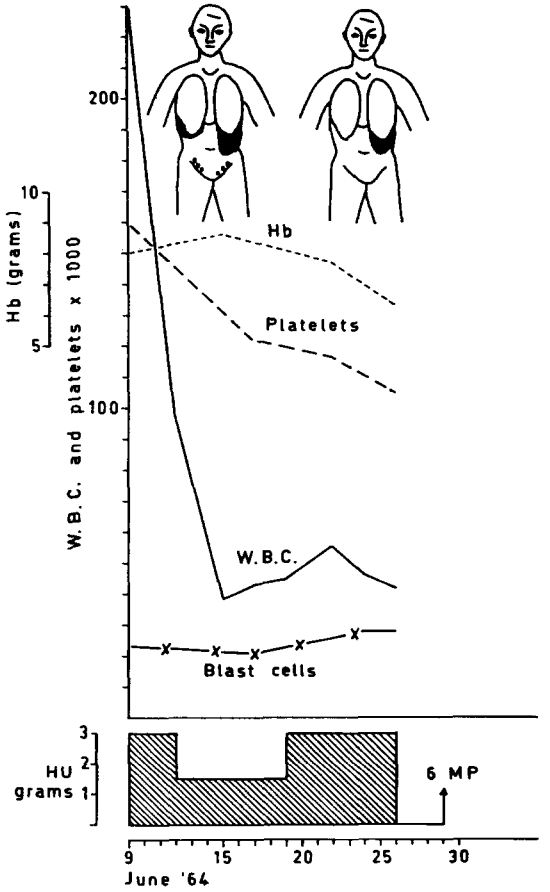

Fig. 2. *Subacute myelogenous leukaemia.*

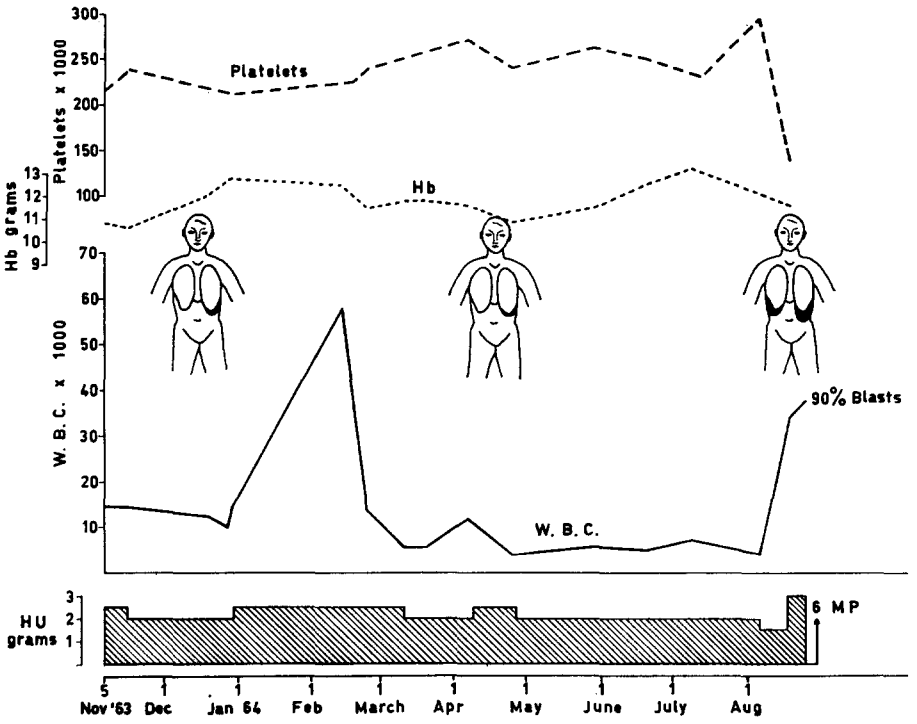

Fig. 3. *Chronic granulocytic leukaemia with subsequent blastic crisis.*

3. Male, age 25 (Fig. 3). The patient had been treated for chronic granulocytic leukaemia for the past 6 years with X-rays and Myleran. The patient had recently become intolerant to the latter drug and had been complaining of nausea, vomiting and other gastro-intestinal disturbances. Treatment with HU was started on a daily dose of 2·5 g, on the 5th of November 1963, at which time the WBC count was 14,500 per mm³ and the differential count showed: Neutrophil polymorphs 79%, Eosinophils 1%, Basophils 1%, Lymphocytes 3%, Myelocytes 15% and Metamyelocytes 1%. Ten days later the dose was reduced to 2 g daily, but increased again on the 1st of January 1964 to 2·5 g daily and maintained at that level for some time, notwithstanding a rise in the WBC count to 57,000 cells per mm³ because of an intercurrent infection. Towards the middle of March, as the WBC count tended to become normal, the dosage of HU was reduced to 2 g. This dose level, with the exception of a brief period, was maintained until the 7th of August. At this time, because the patient was becoming rather leucopenic, the daily dose of HU was cut to 1·5 g. Twelve days later the WBC count rose to 34,000 per mm³ and numerous immature granuloblasts appeared in the peripheral blood. Although the dose of HU was stepped up to 3 g, the WBC count continued to rise and increasing numbers of primitive nucleolated blast cells appeared in the circulation. It was therefore decided to replace HU with 6-mercaptopurine. However, in spite of this change in therapy, the patient progressively deteriorated and died 15 days later.

DISCUSSION

The data obtained from this clinical trial give the impression that hydroxyurea has a definite and selective granulocytopenic effect, while at the same dosage it displays a limited activity on the erythroid and megakaryocytic tissues. In view of these properties, the use of the drug in the treatment of myelogenous leukaemia would seem both feasible and justifiable. However, from the results obtained in the present series of patients, it appears as if the drug gives a favourable therapeutic response mainly in those cases characterized by a fairly differentiated cytological picture. In such cases the drug causes a fairly rapid fall in the WBC count, a decrease or the disappearance in the number of immature granulocytes present in the peripheral blood and a reduction in the size of the spleen. On the credit side, there is also the fact that the compound is effective even in those cases which have been previously treated with radiotherapy or myleran.

On the other hand, even in the treatment of these chronic forms, HU suffers from a number of drawbacks. In the first place the remissions

obtained with HU are shorter-lived than those following treatment with myleran and when the drug is discontinued the leucocyte count promptly rises within a few days; hence the necessity of continuing treatment indefinitely. Secondly, not in all cases, the return of the WBC count to normal levels is associated with a parallel improvement in the red cell count and haemoglobin level. In this context it is worth remembering the megaloblastic changes and the degree of macrocytosis observed in some of our cases. This effect has been repeatedly reported [11] and has been linked with an alteration in the biosynthetic reductive sequence in which ribonucleotide is converted to desoxyribonucleotide [12].

The lack of any beneficial therapeutic effect in the blastic crisis of chronic granulocytic leukaemia and in those cases with a subacute cytological picture "*ab initio*," although the number of patients studied is somewhat restricted, suggests that the drug, at least at the dosage used in our trial, has a limited use in those cases, in which the numbers of nucleolated blast cells in the bone-marrow are even only slightly increased. In addition, the rapid decrease induced by the drug in the number of more mature granulocytes present in the peripheral blood, gives the impression that HU hastens the onset of the blastic crisis in those cases in which such an evolution is foreshadowed by the appearance in the peripheral blood of highly immature blast cells.

Another reason for exerting some caution in the use of HU is the fact that its mechanism of action is still uncertain. The influence exerted by the drug on the conversion of ribonucleotide to desoxyribonucleotide [12] has already been mentioned. The latter authors however consider that the metabolic abnormalities responsible for the megaloblastic changes observed in the bone-marrow of patients treated with HU are probably not related to the cytotoxic activity of the drug. Bendich *et al.* [13] have in fact presented evidence that the antitumour activity of HU consists in a cleavage of DNA.

In conclusion, although HU in our experience does not seem to produce therapeutic responses which are superior to those achieved by Myleran, X-rays, or other antimetabolites, the drug is of interest because it is a member of a new class of agents which operate through a mechanism of action unlike that of other antitumour compounds and therefore encourage the undertaking of future trials with related compounds as they become available.

SUMMARY

Details are given of a clinical trial in which the activity of hydroxyurea (HU) was assessed in a group of patients with myelogenous leukaemia.

In 12 *patients with chronic granulyocytic leukaemia, in the chronic phase of the disease, HU caused a rapid decrease in the number of circulating granulocytes and a reduction in the size of the spleen, while the erythrocyte and platelet levels remained substantially unaffected.*

On the contrary, in 5 *patients in blastic crisis and in one case of subacute myeloblastic-promyelocytic leukaemia, the drug appeared to have a limited use and by acting selectively on the more mature forms of the granulocytic series it seemed to cause a more rapid deterioration of the blood picture.*

The dose levels of HU used in the present trial did not cause any unpleasant side-effects.

Its probable mechanism of action is discussed and future trials with related compounds considered.

RESUME

Cette étude porte sur l'évaluation de l'activité de l'hydroxyurée (HU) dans un groupe de patients souffrant de leucémie myéloïde.

Chez les 12 *malades atteints de leucémie myéloide chronique en phase chronique, la HU a provoqué une diminution rapide du nombre des granulocytes circulants ainsi qu'une réduction des dimensions de la rate, tandis que les taux d'érythrocytes et de plaquettes restaient inchangés.*

Au contraire, chez les patients en période de transformation blastique, et dans un cas de leucémie promyélocytique, myéloblastique subaiguë, le médicament parut avoir une utilité limitée, et, par son action sélective sur les formes plus avancées des séries granulocytiques, parut provoquer une détérioration plus rapide de la formule hématologique.

Les doses de HU employées dans l'essai présent n'ont pas entraîné d'effets secondaires désagréables.

Son mécanisme d'action probable est discuté, et des essais futurs avec des composés proches sont envisagés.

ZUSAMMENFASSUNG

Die vorliegende Arbeit handelt von der klinischen Erprobung der Wirksamkeit des Hydroxy-Harnstoffs bei myeloischer Leukämie.

Bei 12 *Patienten mit chronischer Granulozytenleukämie in der chronischen Phase der Krankheit wurde mit Hydroxy-Harnstoff eine schnelle Verminderung der zirkulierenden Granulozyten und eine Reduzierung der Splenomegalie erzielt, wobei Erythrozyten- und Thrombozytenzahl kaum verändert wurden.*

Im Gegensatz hierzu, schien dieses Medikament bei 5 *Patienten mit blastischen Krisen und bei 1 Fall von subakuter myeloblastischer-promyelozytischer Leukämie nur geringen Einfluss zu haben und dadurch, dass es selektiv auf die reiferen Zellen der Granulozytenserie wirkte, sogar eine Beschleunigung der Verschlechterung des Blutbildes hervorzurufen.*

Die bei diesen Versuchen verwendeten Dosen von Hydro-Harnstoff riefen keine unangenehmen Nebenerscheinungen hervor.

Der wahrscheinliche Wirkungsmechanismus wird diskutiert und zukünftige Anwendung von verwandten Verbindungen werden in Erwägung gezogen.

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Some Aspects of Oestrogen Metabolism in Cases of Human Mammary Neoplasias*

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BREAST cancer and mastopathy are the most important and most frequent lesions of the human mammary gland. Both diseases are never observed prior to puberty and arise almost exclusively in the female. In 1896 the British surgeon Beatson [1] had, for the first time, performed ovariectomies in cases of breast cancer in order to inhibit the malignant growth, and since that time we know that even far-advanced human mammary carcinomas can be forced to regress by removal of the ovaries. Herrell and Dargent [2, 3] reported respectively on 1,900 and 2,000 women who had undergone castration, and found that the breast cancer incidence was only 1·5 per cent in comparison to 15·4 per cent in Herrell's controls which had the same age composition as his breast cancer series. The above-cited simple facts suggest the existence of some pathogenetic conjunction between breast cancer and ovarian function.

Mastopathy is histologically characterized by an excessive fibro-epithelial proliferation and cystic duct ectasias due to obstructive growth, desquamative and secretory matter. There is frequent co-existence of typical mastopathia chronica cystica, fibroadenosis, adenosis and fibroadenomas in the same mammary gland. All these formations are generally regarded as structural variants of the same pathological condition.

Gummel and Wildner [4] from our clinic noted that breast cancer appeared five times as frequently in women with mastopathic lesions as compared with a female control series of the same age composition during the same follow-up period. Similar results of statistic studies were obtained by Warren [5], by Stout [6] of the Presbyterian Hospital in New York and by Kiaer [7] in Copenhagen; they are suggestive that women with mastopathy run a greater risk to develop breast cancer in comparison to women with normal mammary glands. Nevertheless, the vast majority of women with mastopathy do not develop breast cancer.

In many species — from the mouse up to the primates — appropriate, repeated doses of oestrogens lead invariably to mammary lesions containing all of the structural elements characteristic of mastopathy and its variants [8–11]. These proliferations are conditioned ones, disappearing with removal of the oestrogen stimulus.

The influence of oestrogens on the structure of the human mammary gland has not been experimentally investigated. It is, however, well known that therapeutic oestrogen administration [12] and industrial exposure to oestrogens [13] may provoke mastopathy-like proliferations in the male breast in the sense of gynecomastia and even mammary carcinomas.

Thus, from animal experimentation and clinical experience it is likely that abnormal oestrogenic stimulation is an important factor in provoking mastopathy and that mastopathy is a

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fertile soil which may favour the development of cancer of the breast.

In the present study an attempt has been made to determine the excretion pattern of endogenous oestrogen and the metabolic fate of exogenous oestrogen in breast cancer cases with co-existing mastopathy on the one hand and in breast cancer cases without mastopathy on the other. We felt encouraged to perform such a study by our previous findings that the excretory pattern of oestrogens of women suffering from mastopathy differed in a characteristic way from that of women with intact mammary glands [14–16].

Oestrogen excretion patterns in human breast cancer

Comparative estimations of oestrogen excretion were conducted on forty-seven women with breast cancer and co-existing mastopathy and on fifty-six women with breast cancer and no mastopathy. In all cases studied the diagnosis was verified by histological examination of the primary tumour and the surrounding tissue.

Urine was collected on clinical conditions. In pre-menopausal women urine collection period lay between the eighteenth and the twenty-third day of the cycle. From each patient two or three consecutive 24-hr-samples were analyzed for oestrogenic hormones. For oestrogen assay, the improved chemical method of Brown, Bulbrook and Greenwood [17] was applied. By this somewhat exacting and time-consuming procedure the individual oestrogens oestradiol-17β (designed here simply as oestradiol), oestrone and oestriol can be quantitatively determined with reasonable reliability.

The average amounts of the total oestrogen (oestradiol + oestrone + oestriol) excreted per 24 hr were 18·4 μg in group A (breast cancer + mastopathy) and only 13·2 μg in group B ("simple" breast cancer). Mathematical analysis of the results showed that the difference of these mean figures is a significant one and not due to the small age difference of the two groups. Estimations were also made of the excretion of the individual oestrogens. Table 1 shows that the mean amounts of oestradiol and oestrone as proportions of total oestrogen were significantly higher in group A than in group B. Whereas in the cases with breast cancer and mastopathy the average proportion of (oestradiol + oestrone) was computed on approximately 60 per cent of the total oestrogen output, the comparative average figure was only 40 per cent in the "simple" breast cancer cases. At this point it is useful to note that in a series of forty-six healthy women with intact breasts previously examined by us, the average proportion of (oestradiol + oestrone) was 43 per cent of the total oestrogen excretion and thus very similar to that found in patients with "simple" breast cancer.

The excretion patterns varied considerably from one cancer patient to another, within the same group, too. Nevertheless, when each group was taken as a whole, they showed a marked difference in their excretory behaviour for oestrogens: Women with mammary cancer plus mastopathy excreted more of the three classic oestrogens than those with "simple" mammary cancer, and predominantly as oestrone and oestradiol. More details of these findings will be given in a further publication.

Oestradiol loading and oestrogen excretion patterns

It seemed to be of interest to study the metabolism of exogenously administered oestrogens — as reflected by the excretory pattern — in the different groups of breast cancer patients, and to determine whether or not this differs in any way from each other — A tentative scheme for some metabolic inter-relationships of oestrogens is given in Fig. 1.

Each of twelve women of group A and twenty-six women of group B ("blind" random-

Table 1. *Urinary oestrogen excretion patterns in breast cancer patients; mean figures. Group A: breast cancer plus mastopathy. Group B: "simple" breast cancer*

Groups	Oestrogens excreted per 24 hr						
	Average (μg)				Percentage of total		
	($O_1+O_2+O_3$)	O_1	O_2	O_3	O_1	O_2	O_3
A: 47 Pat. (Aver. age 52·1 yrs)	18·4 μg+ (±14·1)	6·4	4·5	7·5	35	25	40++
B: 56 Pat. (Aver. age 56·3 yrs)	13·2 μg+ (±7·4)	3·8	1·6	7·8	29	12	59++

+=$p<0·01$, A: B, χ^2 for trend 7·1.
++=$p<0·01$, [A]O_3%: [B]O_3%, χ^2 for trend 8·1[2]

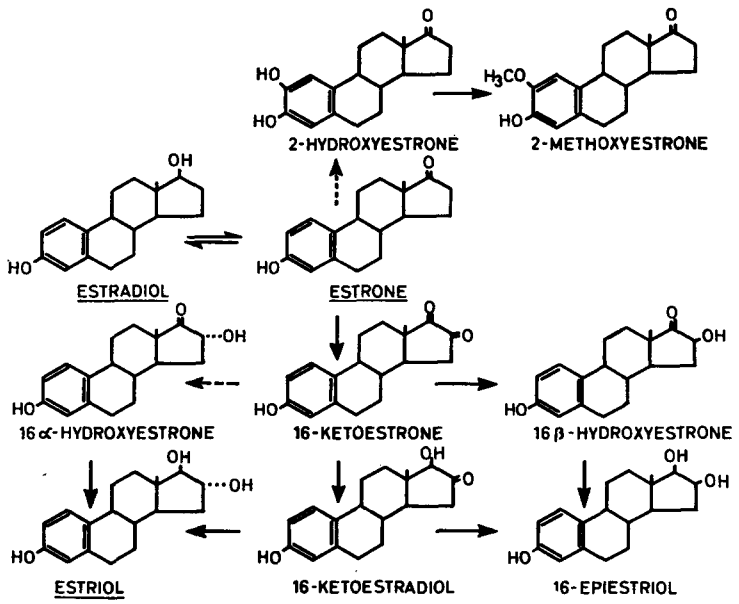

Fig. 1. *Tentative scheme of oestrogen interrelationships.*

ization) received a daily injection of 5 mg of oestradiol-17β-3-monobenzoate in oily solution intramuscularly on three consecutive days. Oestrogen analyses were conducted in a 48-hr-urine-sample pre-experimentally collected and and in another of the second and third day of oestradiol charge.

The increase of the daily total oestrogen output during oestradiol charge showed no differences in the both groups of patients. The mean figures of additional urinary excretion of oestradiol, oestrone and oestriol amounted to 202·1 μg and 211·6 μg per 24 hr respectively in group A and B. There were considerable variations of the output-figures among the subjects studied (Fig. 2, Table 2).

In each case a calculation was made of the percentage of (oestradiol + oestrone) as a proportion of the total oestrogen excretion prior to and during the hormone loading, and of the quantities by which these percentages differed. These difference figures (Δ (O_1 + O_2) %) obtained in the two groups are shown in Table 2. The proportion of (oestradiol + oestrone) of the total oestrogen output increased in each patient of group A. In group B, there were likewise several cases with an increase of the relative excretion of (oestradiol + oestrone), however, other cases of this group were found in which the percentage of the same oestrogens did decrease, and sometimes in a considerable degree. The average increase of the (oestradiol +

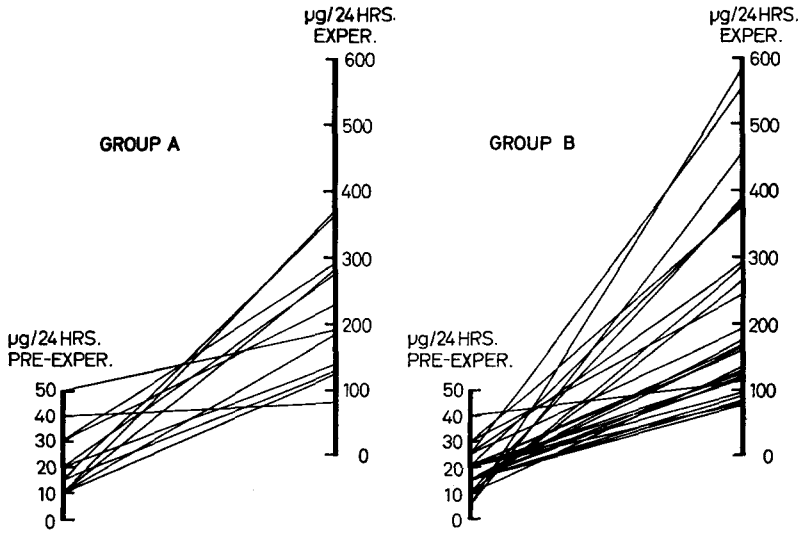

Fig. 2. *Increase of urinary total oestrogen excretion during oestradiol loading.*

Table 2. Individual figures of additional urinary oestrogen excretion during administration of oestradiol-monobenzoate (3 × 5 mg)

Breast cancer plus mastopathy (Group A)		*Breast cancer* (Group B)	
Δ (O_1+O_2)%	Δ($O_1+O_2+O_3$) µg per 24 hr	Δ (O_1+O_2) %	Δ ($O_1+O_2+O_3$) µg per 24 hr
±%		−42	359·2
		−30	456·7
		−22	532·7
		−21	278·2
		−21	258·2
		−21	81·2
		− 4	269·8
		− 3	217·1
+ 1	41·7	+ 1	391·4
+ 1	116·5	+ 2	104·5
+ 8	361·8	+ 5	70·2
+14	255·1	+ 9	168·2
+15	184·0	+13	150·0
+17	351·4	+13	148·0
+20	279·4	+14	80·6
+22	113·5	+20	123·4
+26	122·5	+22	124·1
+37	143·7	+22	113·5
+39	198·5	+24	578·6
+42	257·5	+28	95·6
		+29	157·2
		+29	156·9
		+35	364·5
		+39	73·9
		+41	65·6
		+52	81·8

oestrone) rate during the oestradiol charge was computed to +20·2 per cent for group A and to only +8·6 per cent for group B; logically, the oestriol proportion showed an inverse reaction (Table 3). From our results it is likely that a good deal of breast cancer patients with concomitant mastopathy have a lesser metabolic capacity of converting large amounts of oestradiol to oestriol in comparison to women with simple breast cancer. It may be mentioned here that in both groups during the oestradiol administration the ratio of oestradiol to oestrone shifted in a varying extent to oestradiol.

Testosterone loading and oestrogen excretion patterns

There is evidence that testosterone can be converted to oestrogen in the human [18].

Table 3. Mean figures of additional urinary oestrogen excretion during oestradiol loading

Groups	Oestradiol loading		
	Δ ($O_1+O_2+O_3$) µg/24 hr		Δ (O_1+O_2) %
A: 12 Pat.	202·1	(±100·3)	+20·2
B: 26 Pat.	211·6	(±150·0)	+ 8·6

Administration of testosterone, therefore, generally gives rise to a slight increase of the urinary oestrogen excretion [19]. The conversion of C_{19}-steroids — among them chiefly testosterone, androstenedione, and dehydroepiandrosterone — to oestrogens takes place by the way of 19-hydroxylated intermediates [20–22] (Fig. 3).

In order to pursue the metabolic fate of the additional oestrogens derived from testosterone, oestrogen assays were performed prior to and during testosterone administration to breast cancer patients. To each of fourteen women of group A and twenty-three women of group B ("blind" randomization) daily injections of 100 mg of testosterone propionate in oily solution were given intramuscularly on three consecutive days. Oestrogens were comparatively estimated in two 48-hr-urine samples, collected prior to and during the second and third day of the hormone administration.

The total additional oestrogen output during the testosterone loading amounted on the average to 16·2 µg per 24 hr in group A and to 16·3 µg per 24 hr in group B. Among the subjects examined there were some whose oestrogen excretion remained nearly unchanged and others in which the additional oestrogen

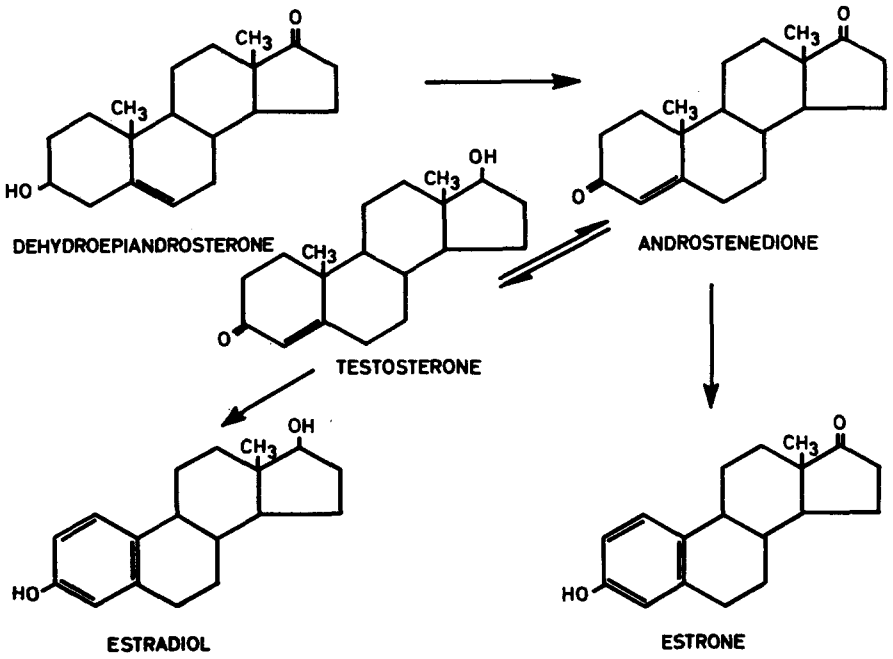

Fig. 3. *Pathway of oestrogen biosynthesis from* C_{19}*-steroids.*

Table 4. *Individual figures of additional urinary oestrogen excretion during testosterone loading*

Breast cancer plus mastopathy (Group A)		*Breast cancer* (Group B)	
$\Delta(O_1+O_2)$ %	Δ $(O_1+O_2+O_3)$ µg per 24 hr	Δ (O_1+O_2) %	Δ $(O_1+O_2+O_3)$ µg per 24 hr
		−44	1·1
		−31	73·8
		−19	18·4
		−10	5·7
		− 6	41·7
−30	36·3	− 5	5·3
−14	22·1	− 4	1·2
− 1	14·4	− 1	21·0
+ 5	38·2	+ 1	17·4
+11	6·6	+ 1	7·3
+12	11·3	+ 1	7·4
+12	6·3	+ 2	25·4
+17	31·7	+ 4	11·2
+21	5·1	+ 5	1·8
+21	25·8	+ 6	5·3
+25	8·8	+ 6	4·2
+28	0·7	+ 7	5·9
+32	4·6	+ 7	5·2
+37	17·1	+16	9·2
		+21	14·2
		+27	4·9
		+30	1·9
		+30	86·7

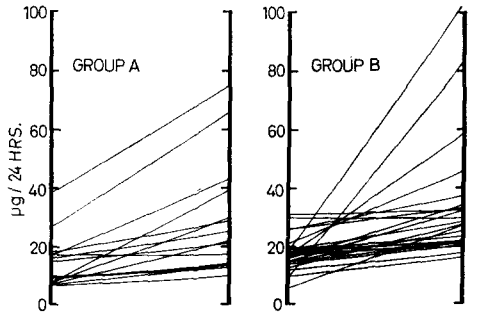

Fig. 4. Increase of urinary total oestrogen excretion during testosterone loading.

surpassed the amount of 35 µg per 24 hr (Fig. 4, Table 4).

The additional quantity of (oestradiol + oestrone) expressed as a percentage of the total oestrogen output showed reactive changes similar to those observed during the oestradiol loading. The mean increase of the rate of (oestradiol + oestrone) amounted to +12·6 per cent in group A (breast cancer with mastopathy) and to +1·9 per cent in group B (simple breast cancer). See Tables 4 and 5.

Table 5. Mean figures of additional urinary oestrogen excretion during testosterone loading

Groups	Testosterone loading	
	Δ ($O_1+O_2+O_3$) µg/24 hr	Δ (O_1+O_2) %
A: 14 Pat.	16·3 (±12·7)	+12·6
B: 23 Pat.	16·2 (±12·5)	+ 1·9

It has been found that a different excretion pattern may result from large doses of oestrogens than from physiologic amounts [23]. Nevertheless, the comparison of the oestrogen excretion patterns during the steroid loadings conducted by us, demonstrates that in certain breast cancer patients with mastopathy the capacity of oestriol formation is diminished. In the general shape, this is valid for either conditions of excessive oestrogen supply and of additional oestrogens endogenously produced in physiologic amounts.

Concluding remarks

Oestradiol and oestrone are the most potent of the naturally occurring oestrogens in stimulating growth of the female reproductive organs, whereas oestriol has largely lost this unique property. For instance, one defined quantity of oestradiol can exert the same proliferative activity as a tenfold quantity of oestrone and a hundredfold quantity of oestriol, as judged by the uterine growth of infantile mice.

It does not appear unreasonable that a similar order of proliferative activity of oestradiol, oestrone and oestriol is valid for the human mammary gland, too, and that a relative increase of oestradiol and oestrone in the urine is reflecting a proportional increase of these oestrogens in the tissue. Under these premisses it might be concluded that in certain women with mammary cancer and co-existing mastopathy a distinct kind of oestrogen stimulation is in existence, based on a reduced or defective metabolic conversion and deactivation of specifically growth-promoting oestrogenic steroids.

RESUME

1. *L'excrétion urinaire de* 17-β-*oestradiol, d'oestrone et d'oestriol a été déterminée chez* 47 *femmes atteintes de cancer mammaire et de mastopathie concomitante* ("*groupe A*", *âge moyen de* 52 *ans*), *et chez* 56 *femmes atteintes de cancer mammaire sans mastopathie* ("*groupe B*", *âge moyen de* 56 *ans*). *Toutes les lésions mammaires de ces cas avaient été vérifiées par examen histologique de la tumeur primaire et du tissu voisin. Pour le dosage des oestrogènes, la méthode chimique selon Brown, Bulbrook et Greenwood a été employée.*

Les valeurs moyennes de l'excrétion d'oestrogènes sont montées à 18,4 µg/24 *heures dans le groupe A, et à* 13,2 µg/24 *heures dans le groupe B. La différence de ces valeurs est significative* ($p<0{,}01$; χ^2-*test*) *et n'est pas explicable par la différence d'age des groupes. La proportion moyenne oestradiol+oestrone a été calculée à* 60 *pour cent de l'excrétion totale d'oestrogènes dans le groupe A, et à* 40 *pour cent seulement dans le groupe B.*

2. *Douze femmes du groupe A et* 26 *femmes du groupe B ont été soumises à un test de surcharge avec oestradiol (injections intramusculaires quotidiennes de* 5 *mg d'oestradiol-benzoate pendant trois jours consécutifs). L'augmentation de l'excrétion totale d'oestrogènes, pendant le test de surcharge, était presque identique dans les deux groupes examinés* (202 µg/24 *heures dans le groupe A et* 211 µg/24 *heures dans le groupe B). La surcharge d'oestradiol provoquait une augmentation marquée de la proportion oestradiol+oestrone, de* 20,2 *pour cent dans le groupe A, tandis que dans le groupe B, la valeur correspondante augmentait de* 8,6 *pour cent seulement.*

3. *Quatorze femmes du groupe A et* 23 *femmes du groupe B ont reçu des injections intramusculaires quotidiennes de* 100 *mg de propionate de testostérone, pendant trois jours consécutifs. Pendant cette surcharge hormonale, l'excrétion additionnelle totale d'oestrogènes s'élevait en moyenne à* 16,2 µg/24 *heures dans le groupe A, et à* 16,3 µg/24 *heures dans le groupe B. On observa une augmentation moyenne de la proportion oestradiol+oestrone, de* 12,6 *pour cent dans le groupe A, et de* 1,9 *pour cent dans le groupe B.*

4. *Les résultats présentés semblent indiquer qu'un grand nombre de femmes attentes de cancer mammaire et de mastopathie concomitante aient une capacité métabolique moindre dans la conversion d'oestradiol et d'oestrone exogène ou endogène en oestriol, par comparaison avec les femmes atteintes de cancer mammaire simple.*

SUMMARY

1. *The urinary excretion patterns of oestradiol-*17β, *oestrone and oestriol were estimated on forty-seven women with breast cancer and co-existing mastopathy ("group A", average age* 52 *years) and on fifty-six women with breast cancer and no mastopathy ("group B", average age* 56 *years). All mammary lesions of the cases studied were verified by histological examination of the primary tumour and the surrounding tissues. For oestrogen assays, the chemical method described by Brown, Bulbrook and Greenwood was employed.*

The mean amounts of the total oestrogen excreted per 24 *hr were* 18·4 µg *in group A and* 13·2 µg *in group B. The difference of these figures is a significant one* ($p < 0{\cdot}01$; χ^2*–test) and not due to the age difference of the groups. The average proportion of (oestradiol + oestrone) was computed on* 60 *per cent of the total oestrogen output in group A and on only* 40 *per cent in group B.*

2. *Twelve women of group A and twenty-six women of group B were submitted to an oestrogen loading test (daily injections of* 5 *mg oestradiol benzoate i.m. on* 3 *consecutive days). The increase of the total oestrogen excretion during the test was nearly identical in both groups studied* (202 µg/24 *hr in group A and* 211 µg/24 *hr in group B). Oestradiol loading caused a marked augmentation of the (oestradiol + oestrone) proportion by* 20·2 *per cent in group A, whereas in group B the corresponding figure increased by* 8·6 *per cent only.*

3. *To each of fourteen women of group A and twenty-three women of group B daily injections of* 100 *mg of testosterone propionate were given i.m. on three consecutive days. During this hormone loading, the total additional oestrogen excretion amounted on the average to* 16·2 µg/24 *hr in group A and* 16·3 µg/24 *hr in group B. There was a mean increase of the rate of (oestradiol + oestrone) by* 12·6 *per cent in group A and by* 1·9 *per cent in group B.*

4. *From the present study it is likely that a good deal of breast cancer patients with co-existing mastopathy have a lesser metabolic capacity of converting endogenous or exogenous oestradiol and oestrone to oestriol in comparison with women suffering from "simple" breast cancer.*

ZUSAMMENFASSUNG

1. *Das Ausscheidungsmuster von* 17-β-*Ostradiol, Östron und Östriol im Urin wurde bei* 47 *Frauen mit Brustkrebs und begleitender Mastopathie ("Gruppe A", Durchschnittsalter* 52 *J.) und bei* 56 *Frauen mit Brustkrebs ohne Mastopathie ("Gruppe B", Durchschnittsalter* 56 *J.) bestimmt. Die Mammaveränderungen wurden in allen Fällen durch histologische Untersuchungen des Primärtumors und des umgebenden Gewebes*

verifiziert. Für die Östrogen-Bestimmungen wurde die chemische Methode von Brown, Bulbrook und Greenwoodangewendet.

Die Mittelwerte der Gesamt-Östrogenausscheidung pro 24 Stunden betrugen 18,4 µg in Gruppe A und 13,2 µg in Gruppe B. Die Differenz der beiden Werte ist signifikant ($p < 0{,}01$; χ^2-Test) und beruht nicht auf dem Altersunterschied der Gruppen. In Gruppe A errechnete sich der Anteil von (Östradiol + Östron) im Mittel auf 60 Prozent der Gesamt-Östrogenausscheidung und in Gruppe B auf nur 40 Prozent.

2. *12 Frauen der Gruppe A und 26 Frauen der Gruppe B wurden einem Östrogen-Belastungstest unterzogen (tägliche Injektionen von 5 mg Östradiolbenzoat i.m. an 3 aufeinanderfolgenden Tagen). Der Anstieg der Gesamt-Östrogenausscheidung während des Tests war in beiden Gruppen nahezu identisch (Gruppe A: 202 µg/24 Std., Gruppe B: 211 µg/24 Std.). Die Östradiolbelastung bewirkte eine deutliche Zunahme des (Östradiol + Östron)-Anteils um 20,2 Prozent in Gruppe A, wohingegen in Gruppe B der entsprechende Wert nur um 8,6 Prozent wuchs.*

3. *14 Frauen der Gruppe A und 23 Frauen der Gruppe B erhielten tägliche Injektionen von 100 mg Testosteronpropionat i.m. an 3 aufeinanderfolgenden Tagen. Unter dieser Hormonbelastung belief sich die zusätzliche Gesamt-Östrogenausscheidung im Mittel auf 16,2 µg/24 Std. in Gruppe A und 16,3 µg/24 Std. in Gruppe B. Der mittlere Anstieg des (Östradiol + Östron)-Anteils lag bei 12,6 Prozent in Gruppe A und bei 1,9 Prozent in Gruppe B.*

4. *Die vorliegenden Untersuchungen deuten darauf hin, daß ein beträchtlicher Anteil der Brustkrebspatienten mit begleitender Mastopathie eine geringere metabolische Fähigkeit besitzt, endogenes oder exogenes Östradiol und Östron in Östriol umzuwandeln, als Frauen mit "einfachem" Brustkrebs (ohne Begleitmastopathie).*

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Notes et Communications

Groupe Européen de Chimiothérapie Anticancéreuse (G.E.C.A.)

Protocole pour les Essais Cliniques de Traitements des Leucémies et Hématosarcomes

I. Introduction

Le Groupe Européen de Chimiothérapie Anticancéreuse conduit des essais sur la valeur thérapeutique de nouveaux produits dans les leucémies et hématosarcomes.

Les produits étudiés peuvent être synthétisés parle groupe ou lui avoir été soumis, soit pour étude expérimentale et clinique, soit seulement pour étude clinique.

Le responsable de la section "leucémies" est le Professeur agrégé G. MATHÉ. Est enfin attaché au GECA un groupe coopérateur "Leucémies et Hématosarcomes" présidé par le Professeur G. MARCHAL.

Les essais actuellement prévus ne concernent que des traitements aigus, c'est-à-dire des traitements des phases évolutives. On étudie donc l'effet des produits, d'une part, sur les symptômes, d'autre part, sur l'évolution de la phase évolutive.

La section "leucémie" n'envisage pas d'engager actuellement des essais de traitements chroniques, c'est-à-dire de traitements continus ou discontinus appliqués pendant de longues périodes et dont on étudie l'effet sur la survie. Seul le groupe coopérateur "Leucémies et Hématosarcomes" conduit de tels essais.

II. But et divers temps des essais de traitements aigus

Lorsque le "screening" expérimental a montré qu'un produit possède une activité anticancéreuse, les questions suivantes se posent, auxquelles les essais cliniques doivent tenter de répondre:

A. Première question: Le produit est-il efficace dans telle maladie?

B. Les questions suivantes dépendent de l'origine du produit et de sa structure chimique.

   1. Si le produit est d'une structure chimique à laquelle appartiennent d'autres substances déjà utilisées en clinique, deux questions se posent:

      a. Le nouveau produit est-il efficace chez des malades résistants à un autre ou aux autres produits de la même série chimique?

      b. Le nouveau produit est-il plus efficace que le meilleur des autres de cette même série?

   2. Si le produit n'appartient pas à une série dont on utilise déjà d'autres substances, la question qui se pose est la suivante : quelle qest l'efficacité du produit?

III. Description des divers temps des essais de traitements aigus, nombre de malades et témoins

A. Premier temps

   Le produit est-il efficace contre telle maladie? La substance est administrée

chez tous les malades au fur et à mesure qu'ils se présentent, sauf s'ils sont résistants à un éventuel produit de la même série, et sauf contre-indication d'un autre ordre, sur laquelle nous reviendrons. Le produit est considéré comme efficace s'il s'est montré notablement actif, c'est-à-dire s'il induit une rémission apparement complète ou une rémission incomplète (voir plus loin la terminologie utilisée pour désigner la qualité des résultats) chez au moins un sujet.

L'essai s'arrête donc au premier sujet présentant une telle rémission. Cependant, si le produit est très peu actif ou inactif, l'essai risque de continuer indéfiniment On l'arrêtera de toute manière si on n'a pas obtenu de résultats pour 15 malades successifs.

Le nombre n=15 a été déterminé de la manière suivante: on a, au maximum, 10 chances sur 100 de laisser échapper un produit qui serait actif 15 fois sur 100.

On notera que l'essai ne demande pas de témoins (on comparera cependant les périodes évolutives traitées à une période témoin de même longueur précédant immédiatement la période évolutive traitée) et n'exige qu'un petit nombre de sujets; son objet est surtout d'éliminer rapidement des produits inactifs ou très peu actifs; mais des produits peu actifs ne sont pas éliminés, et il reste nécessaire de chiffrer l'efficacité de ces produits par les temps ultérieurs.

B. Temps ultérieurs

1. Le produit est d'une série chimique à laquelle appartiennent des produits antérieurement disponibles et utilisés.

(a) Deuxième temps. Le produit est-il efficace chez des malades résistants aux substances de ce groupe?

Le produit est administré à des patients que l'on aura jugés résistants à une des substances du groupe chimique; par exemple, si le nouveau produit à tester est un antimétabolite du groupe des antipurines, il ne sera administré qu'à des malades résistants à la 6-Mercaptopurine ou à une autre antipurine.

Comme dans le temps précédent, le produit est considéré comme efficace s'il s'est montré notablement actif, c'est à dire capable d'induire une rémission apparement complète ou une rémission incomplète chez un sujet au moins; s'il n'en induit pas, on limitera à 15 le nombre des sujets testés.

(b) Troisième temps

Ce troisième temps n'est pas obligatoire. Il consiste à étudier si un produit est plus efficace qu'un autre de la même série chimique antérieurement disponible et précédement utilisé dans la maladie à l'étude.

Il nécessite la comparaison de 2 groupes semblables, selon un protocole détaillé qui sera étudié en temps voulu en fonction des données des temps précédents.

2. Le produit n'est pas d'une série à laquelle appartiennent des produits antérieurement disponibles.

Le second temps consiste alors à étudier le pourcentage d'activité du produit. Il nécessite une série de malades recevant le produit sans groupe témoin. Le nombre de ces malades dépend de la précision recherchée dans l'énoncé de l'efficacité, selon le barème actuellement à l'étude pour réduire au maximun le nombre de sujets nécessaires.

C. Utilisation simultanée de divers produits.

Les considérations exposées plus haut sur les buts et temps des divers traitements conduisent à considérer:

1. 2 types de produits: les produits "nouveaux" et ceux qui appartiennent à une série chimique efficace.

2. 4 types de malades: ceux qui sont "neufs", neufs pour une série chimique, partiellement résistants pour les produits connus de cette série, totalement résistants à ces produits.

Le tableau ci-après indique que les malades peuvent être soumis à l'essai pour les deux types de produits.

Malades	Produits	
	Nouveaux	D'une série S
Nouveaux	Temps 1 et 2	Temps 1
Nouveaux pour S	Temps 1 et 2	Temps 1
Partiellement résistants à S	Temps 1 et 2	
Résistants à S	Temps 1 et 2	Temps 2

L'examen de ce tableau montre que la meilleure politique consiste à avoir toujours en essai simultanément 2 produits au moins, l'un nouveau, l'autre d'une série S.

Pendant que le produit d'une série S est au temps I, on expérimente ce produit par priorité sur les malades nouveaux ou nouveaux pour S, et les malades des deux autres catégories sont soumis au traitement nouveau.

Quand un produit d'une série S est admis au Temps 2, on l'expérimente par priorité sur les malades totalement résistants à S, les malades des 3 autres catégories étant soumis au traitement nouveau.

L'admission d'un produit d'une série S au temps 3 n'est pas envisagée pour le moment.

En fait, il est intéressant d'étudier simultanément plus de 2 produits, les malades résistants à l'un d'eux pouvant être soumis à l'un des autres.

IV. Choix des malades soumis aux essais

Ne sont soumis aux essais que des malades présentant des lésions objectives et mesurables, que ces lésions soient physiques, par exemple une hyperthrophie ganglionnaire ou splénique ou cutanée, ou qu'elles soient hématologiques, par exemple le passage dans le sang ou la présence dans la moelle de cellules anormales.

Lorsque le symptôme physique consiste en l'hypertrophie d'un organe non hématopoïétique, il est exigé une biopsie ou une ponction pour prouver que cette hypertrophie est liée à la prolifération des cellules en cause dans la maladie.

On ne tient compte de modifications de désordres chimiques ou biologiques non spécifiques (telle que la vitesse de sédimentation, l'augmentation de la lacticodéshydrogénase) que s'il existe en outre des modifications des désordres cliniques ou hématologiques.

On peut soumettre à l'essai, des malades sous traitement par un autre produit, à condition que, pendant la période témoin, ce traitement se soit révélé inefficace, et à condition qu'on puisse ou en diminuer la dose ou en interrompre l'administration pendant la période d'essai du nouveau produit. Cette situation peut se présenter chez des malades traités par la cortisone, et que l'on ne peut priver de cette substance que si l'on peut agir sur leur maladie par un autre produit.

Il est bien évident que l'on ne peut soumettre aux essais thérapeutiques des malades présentant une forme de maladie sur laquelle on sait que d'autres thérapeutiques, telle que l'irradiation locale, donnent des résultats excellents, ce qui rendrait immoral de priver le malade d'une telle thérapeutique en raison d'un essai alléatoire.

V. Nomenclature des affections soumises aux essais

La nomenclature utilisée est celle actuellement à l'étude dans le centre international de référence de l'Organisation Mondiale de la Santé pour les leucémies et hématosarcomes dont le responsable est le professeur ag. G. MATHÉ.

1. Leucémies et autres maladies affectant le sang périphérique

Aiguës
- Leucémie aiguë lymphoblastique
- Leucémie aiguë myéloblastique
- Leucémie aiguë promyélocytaire
- Leucémie aiguë monoblastique
- Leucémie aiguë à cellules monocytoïdes (de Naegeli)

Chroniques
- Leucémie lymphocytaire chronique
- Leucémie myéloïde chronique
- Leucémie histiocyto-monocytaire
- Leucémie myélo-monocytaire
- Polyglobulie de Vaquez
- Splénomégalie myéloïde idiopathique

Rares
- Erythromyélose aiguë de di Guglielmo
- "Leucémie" à mégacaryocytes et (ou) thrombocytémie idiopathique
- Leucémie à éosinophiles
- Leucémie à basophiles
- Mastocytose maligne
- Leucémie à plasmocytes

2. Hématosarcomes non leucémiques et affections voisines

Réticulo- et histiocytosarcomes
- Réticulosarcome
- Histioblastosarcome
- Histiocytosarcome

Lymphosarcomes
- Lymphoblastosarcome
- Lymphocytosarcome

Myélome et macroglobulinémie de Waldenström
- Myélome
- Macroglobulinémie de Waldenström

Lymphogranulome de Hodgkin
- Granulome de Hodgkin
- Paragranulome de Hodgkin
- Sarcome de Hodgkin
- Syndrome macrofolliculaire de Brill-Symmers
- Mycosis fongoïde

3. Formes mixtes et intermédiaires.

VI. Méthodes générales de travail

Autant que possible, on obtient les documents capables de représenter les lésions: photographies macroscopiques, radiographies. Les lésions sont mesurées.

Les documents sont étudiés par les 4 membres de la section réunie.

On ne tient compte, en principe, que des modifications de lésions objectives. Les modifications des symptômes subjectifs ne sont interprétées que comme renseignements complémentaires à ceux fournis par les modifications des lésions objectives.

On s'efforce d'obtenir des renseignements non seulement sur des lésions facilement accessibles, par exemple des lésions périphériques, mais encore sur des lésions profondes, en particulier abdominales: la lymphographie abdominale est pratiquée systématiquement.

VII. Modalité d'administration des produits

1. La dose quotidienne et la voie d'administration sont désignées par l'étude toxicologique des produits.

   Pour la plupart d'entre eux, l'administration est quotidienne et l'on pratique des contrôles hématologiques régulièrement, par exemple toutes les semaines; pour d'autres produits, l'administration est seulement hebdomadaire, ou tous les 3 ou 5 jours, et l'on pratique un examen hématologique avant chaque injection; pour d'autres produits enfin, on administre la substance quotidiennement pendant 7 jours, puis on laisse le malade 7 jours au repos et l'on pratique alors un contrôle hématologique; on recommence une administration de 7 jours.
2. La dose totale n'est pas fixée à l'avance. Après une certaine dose cumulative, on juge de l'effet: (a) en l'absence d'effet, on cesse avant la manifestation de la toxicité; (b) si l'effet désiré est obtenu, on peut cesser; si un effet non complet mais notable est obtenu, on poursuit jusqu'à la dose maximale tolérée.

VIII. Terminologie utilisée pour désigner les résultats obtenus

Les résultats sont classés dans les quatre possibilités suivantes:

1. Rémission apparement complète

Ce terme signifie qu'il n'existe plus aucune manifestation clinique, hématologique (y compris dans la moelle osseuse étudiée par ponction sternale), ni chimique de la maladie.

2. Rémission incomplète

La phase évolutive a cédé, mais tous les symptômes n'ont pas disparu.

3. Echec non total

Ce terme signifie que la phase évolutive se poursuit malgré la régression de quelques manifestations.

4. Echec total

   Aucun effet n'a été enregistré.

On considère que l'effet est notable lorsqu'on a obtenu une rémission apparemment complète ou une rémission incomplète.

IX. Activité générale de la section

Lorsqu'un temps est terminé, ou lorsqu'on désire commencer un nouveau temps, la section avertit le groupe.

Les résultats sont discutés au cours d'une réunion du groupe.

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Announcements

Symposium International sur le Cancer Mammaire Avancé à Lyon, France

CE SYMPOSIUM aura lieu du 5 au 7 mai 1966 au Palais des Congrès à Lyon. Il est placé sous les auspices de la Société Nationale Française du Cancer et de l'Union Internationale.

Le programme comprend les sujets suivants

Le 5 mai: Surrénalectomie et hypophysectomie : techniques et résultats.

Le 6 mai : Aspects cliniques et biologiques.

Le 7 mai : Evaluation et comparaison statistiques avec les autres thérapeutiques.

Des participants de la plupart des pays européens, des Etats-Unis, du Canada et de l'Inde sont déjà inscrits.

Les communications seront limitées à 15 minutes : elles seront suivies d'une discussion. Les langues utilisées seront le français et l'anglais avec traduction simultanée.

Comité d'organisation

Professeur DARGENT, Président
(Directeur du Centre Léon Bérard).
Docteur JURET, Secrétaire Général
(Institut Gustave Roussy).
Docteur ROMIEU
(Centre Anticancéreux de Montpellier).
Docteur SARRACINO
(Centre René Huguenin).
Mademoiselle ROUQUETTE.

Comité de Lyon

Docteurs COLON, DUTOU, MAYER, POMMATOU.

Pour de plus amples renseignements, écrire à :

Monsieur le Docteur DUTOU.
Centre Léon Bérard,
28, rue Laënnec,
LYON 8e.

Droit d'inscription : 30 FF.

International Symposium on Major Endocrine Surgery in Advanced Cancer of the Breast

FROM May 5–7, 1966, in Lyon, France, at the "Palais des Congrès" sponsored by the National Cancer Society and the International Union against Cancer.

Preliminary program

May 5 : Adrenalectomy and hypophysectomy : technics and results.

May 6 : Clinical and biological aspects.

May 7 : Statistical evaluation and comparison with other therapies.

The following countries are represented at the meeting:

Belgium	Ireland
Canada	Italy
France	Poland
Germany	Rumania
Great Britain	Sweden
Holland	Switzerland
India	U.S.A.

Communications will be limited to 15 minutes, followed by a discussion. Languages will be French and English with simultaneous translation.

Organization Committee

Professor DARGENT, President
(Director of Léon Bérard Centre).
DR. JURET, General Secretary
(Gustave Roussy Institute).
Dr. ROMIEU
(Anti-Cancer Centre of Montpellier).
Dr. SARRACINO
(René Huguenin Centre).
Miss ROUQUETTE.

Lyon Committee

Doctors COLON, DUTOU, MAYER, POMMATOU.

For further information, write to:

Dr. DUTOU,
Centre Léon Bérard,
28, rue Laënnec,
LYON 8e. (69).

Registration fee : 30 French francs.

Europ. J. Cancer. Vol. 2, p. 91. Pergamon Press 1966. Printed in Great Britain.

Errata

E. CIARANFI, L. LORETI, A. BORGHETTI and G. G. GUIDOTTI: Studies on the anti-tumour activity of aliphatic aldehydes—II. Effects on survival of Yoshida ascites hepatoma-bearing rats, *Europ. J. Cancer* **1,** 147–151 (1965).

Page 149, column 1, line 13

for "*cell and large in volume. In these animals a*"

read "*cells with marked morphological alterations*".

R. DOURMASHKIN: An electron microscopic study of Burkitt tumour biopsies, *Europ. J. Cancer* **1,** 309–312 (1965).

Caption to Fig. 3 (facing page 310), line 2

for "*portions of H3 degenerating cells*"

read "*portions of 3 degenerating cells*".

Europ. J. Cancer Vol. 2, pp. 93-103. Pergamon Press 1966. Printed in Great Britain

Cultures Organotypiques de Longue Durée de Deux Tumeurs Humaines du Tube Digestif

ETIENNE WOLFF et EMILIENNE WOLFF

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Collège de France

Place Marcelin Berthelot, Paris Ve

LE PRÉSENT mémoire donne une vue d'ensemble des résultats récents que nous avons obtenus sur la culture organotypique *in vitro* de tumeurs humaines. Nous avons eu pour but de maintenir en vie, hors de l'organisme, des tumeurs malignes prélevées directement sur le patient, en conservant leur structure et leur pouvoir de multiplication. Les cultures *organotypiques* de cancers diffèrent profondément des cultures de cellules (ou cultures *histiotypiques*) qui ont été obtenues dans de nombreux laboratoires et qui constituent actuellement une méthode de routine pour de nombreuses recherches. Les cultures cellulaires ne permettent de conserver ni l'organisation tissulaire, ni la différenciation cellulaire des tumeurs. Leurs cellules sont en général dédifférenciées, elles s'étalent en couches monocellulaires ("monolayers"), sur la paroi des récipients.

Au contraire, la culture organotypique tend à conserver les structures organisées, la différenciation cellulaire, les propriétés physiologiques des tumeurs et à reconstituer des nodules massifs, qui se multiplient dans les trois dimensions de l'espace. Ainsi nous avons obtenu de *véritables souches organotypiques*, qui paraissent devoir se multiplier indéfiniment, de la même manière que les souches cellulaires, qui sont actuellement utilisées dans de nombreux laboratoires.

De telles souches organotypiques doivent permettre une expérimentation aisée sur des nodules de petites dimensions, dont la structure et les propriétés sont les mêmes que celles de la tumeur initiale. Ainsi l'expérimentation sur des tumeurs humaines devient possible. D'autre part, les conditions de nutrition de ces tumeurs peuvent nous renseigner sur les facteurs qui permettent aux tumeurs de proliférer *in vitro* comme dans l'organisme humain. C'est essentiellement vers ce problème que nos recherches sont actuellement orientées.

Rappelons que c'est en 1956 que nous avons entrepris des recherches sur la culture organotypique de tumeurs *in vitro*. Nous avons réussi successivement, avec nos collaborateurs, à cultiver:

1 — Des tumeurs de souris et de rat [1 – 5].

2 — Des souches cellulaires humaines telles HeLa, KB, FOGH, qui reconstituent *in vitro* des nodules massifs et organisés [6 – 10].

3 — Des tumeurs humaines prélevées directement sur des malades, au cours d'opérations chirurgicales. De nombreuses tumeurs ont été explantées en primocultures. Plusieurs d'entre elles ont donné lieu à des cultures de longue durée [11 – 18].

I. TECHNIQUE — PRINCIPES GENERAUX

Comme la technique a donné lieu à des modifications et améliorations successives, qui ont été publiées d'une manière échelonnée, nous indiquerons ici en détail les étapes et les différentes modalités de la technique.

Mais il faut souligner dès l'abord que *les milieux de culture qui se sont montrés favorables à la culture d'organes embryonnaires* [19] *ne suffisent pas à la culture de tumeurs.* La raison pour laquelle il en est ainsi apparaîtra au cours de cet article: *il manque à ces milieux certaines substances stimulatrices de la croissance cancéreuse.* Aussi les cultures organotypiques de tumeurs n'ont-elles d'abord réussi que lorsqu'on associait les explants cancéreux à des explants d'organes d'embryons de poulet, plus particulièrement au mésonéphros. Ce sont donc des cultures au 2ème degré, le mésonéphros se nourrissant du milieu et la tumeur maligne du mésonéphros. Dans les cultures directes sur le mésonéphros, la tumeur vit en parasite aux dépens des organes embryonnaires qu'elle détruit et supplante progressivement. Dans les cultures où une membrane est interposée entre le mésonéphros et la tumeur, celle-ci se nourrit de substances qui sont sécrétées par l'organe et filtrent à travers la membrane.

Enfin, dans nos dernières recherches, nous avons pu substituer au substratum vivant des extraits de levure, dont le mode de préparation sera indiqué dans les pages suivantes.

Le milieu de base

Ce milieu a servi d'abord aux cultures d'organes embryonnaires qui ont été utilisés comme substratum alimentaire des cultures de tumeurs. La composition du milieu est la suivante [10]:

1. Solution d'agar agar à 1% dans du liquide de Gey — 12 vol.
2. Extrait d'embryon de poulet de 8 jours 1/2 — 5 vol.
3. Sérum de cheval — 5 vol.
4. Pénicilline: 1 gouttelette contenant — 10 à 15 unités Oxford

On prépare d'abord la gélose (Difco) à raison de 10 g pour 1000 g de liquide de Gey. On la fait bouillir à 100°. On la répartit dans de petits ballons de 50 cm³, on les stérilise à l'autoclave. Au moment de la préparation des milieux, on réchauffe les ballons à une température suffisante pour que la gélose devienne un liquide homogène et clair (environ 80°).

On dispose en outre de tubes à essais à demi remplis d'une solution de Tyrode stérile. [Celle-ci a été stérilisée en deux parts: une solution contenant tous les constituants, moins le bicarbonate (950 g), une solution contenant le bicarbonate (150 g)]. Ces deux parts sont mélangées à froid au moment de l'emploi.

On a préparé un extrait d'embryons de Poulet de 8 à 9 jours dilué de moitié dans le liquide de Tyrode. On dispose cet extrait dans des tubes à essais.

On place côte à côte, dans un porte tube incliné, un tube de gélose, un tube de sérum de cheval, un tube d'extrait d'embryon, un tube d'une solution de pénicilline (celle-ci peut aussi être incorporée au liquide de Tyrode avant la confection des milieux). Une pipette laissant tomber des gouttes d'environ 1/10 cm³ est placée dans chacun des trois premiers tubes (il est nécessaire que les trois pipettes soient calibrées aux mêmes dimensions), une pipette capillaire, laissant tomber une goutte correspondant à la dose convenable de pénicilline est placée dans le 4e tube.

Les milieux sont préparés dans des récipients de verre massif à section carrée, présentant une cavité hémisphérique d'une contenance de 5 cm³, appelés godets pour sérologie, et plus communément "salières". Le volume de la cavité est de 5 cm³. On prépare un milieu complet de culture successivement dans chaque salière: on verse d'abord, avec la première pipette, la solution de gélose, encore très chaude, qui se refroidit au contact du verre; on verse, avec la 2e pipette, le sérum, avec la 3e, l'extrait d'embryon, avec la 4e la pénicilline. On mélange intimement les constituants à l'aide d'un agitateur. Toutes ces opérations doivent être faites très rapidement, afin que la gélose reste fluide. Il est nécessaire qu'elles soient faites dans l'ordre indiqué, afin que la température du mélange soit progressivement abaissée, de telle sorte que ni le liquide de Tyrode ni l'extrait d'embryon ne soient altérés par une température trop élevée. On laisse le milieu se solidifier dans les récipients qu'on a recouverts d'une plaque de verre carée couvrant exactement les bords de la salière (Fig. 1). Il est nécessaire que le milieu soit parfaitement homogène, et sa surface parfaitement lisse.

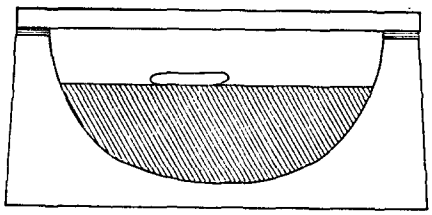

Fig. 1. Schéma général d'une culture organotypique. Un explant est déposé à la surface du milieu (en hachures) solidifié par un gel d'agar. Le couvercle de verre est luté à la paraffine sur le récipient, dont l'atmosphère est saturée d'humidité.

C'est à la surface, et non au sein du milieu que les explants sont déposés avec précaution. Ils ne doivent pas être enfoncés dans le milieu et il faut prendre garde de ne pas égratigner la surface de la gélose, pour éviter que des crevasses ou des dépressions ne s'emplissent de liquide. L'explant ne doit pas être immergé, même partiellement, dans du liquide. On doit le sécher avec soin à l'aide de petits morceaux de papier filtre, sous le microscope binoculaire — car l'organe ou le fragment d'organe est généralement détrempé par le liquide de Tyrode dans lequel ont été faits la dissection et le prélèvement. Des traces de liquide favorisent en effet la migration et la prolifération des cellules hors de l'explant, et nuisent à l'intégrité de celui-ci. On doit assécher les explants aussi souvent que cela est nécessaire, en moyenne tous les deux jours.

Après la mise en culture des organes, les lames de verre qui servent de couvercle sont lutées très soigneusement à l'aide d'un pinceau avec un mélange stérile de cire et de paraffine très chaudes. Il est nécessaire que cette fermeture soit étanche, car l'atmosphère du récipient de culture doit être saturée d'humidité. C'est à ce prix que les explants peuvent supporter d'être cultivés à la surface du milieu. Ainsi, par leur surface inférieure, en contact avec le milieu, ils absorbent les substances nutritives; par le reste de leur surface ils respirent l'air atmosphérique de la chambre de culture.

Des transferts sur de nouveaux milieux doivent être effectués dans le cas de cultures de longues durées. En pratique, il suffit d'absorber soigneusement avec du papier filtre le liquide qui tend à se former autour de l'explant, pour éviter le danger de l'immersion et pour éliminer les produits de désassimilation. On peut aussi déplacer l'organe sur le milieu.

On place alors les récipients de culture sur de longues planchettes de bois présentant deux rebords longitudinaux. La largeur de la planchette est légèrement supérieure au côté des récipients. Le fond de la planchette est percé d'évidements qui permettent la circulation de l'air dans les couveuses. Les planchettes sont alors placées dans des incubateurs réglés à 37° ou 38° 5, suivant les besoins.

Technique de la membrane vitelline

Une modification importante de la méthode a consisté à déposer l'organe sur une très fine membrane naturelle: la membrane vitelline du jaune d'oeuf de poule non incubé. Ce dispositif s'est révélé très favorable aux cultures de tumeurs sur des organes embryonnaires [10]. Deux modalités de ce dispositif ont été employées:

1. Dans un premier cas, une membrane vitelline est interposée entre le milieu et les explants. Pratiquement, on enveloppe ceux-ci dans les plis d'une membrane vitelline comme dans une pochette. On procède de la manière suivante: on prélève la membrane vitelline, on la découpe en fragments rectangulaires qu'on débarrasse soigneusement du vitellus et de l'albumine. On les lave longuement dans le liquide de Tyrode. Puis on place un morceau rectangulaire sur le milieu, le côté vitellin en haut. On dispose ensuite les explants sur la membrane vitelline, comme on le faisait précédemment sur le milieu nu. Puis on saisit l'un des bords libres de la membrane avec des pincettes d'horloger et on le replie par-dessus les explants. Ainsi les fragments d'organes ou de tumeurs, auxquels ce procédé est particulièrement favorable, sont insérés dans une poche de la membrane à laquelle ils adhèrent par leurs surfaces inférieure et supérieure (Fig. 2). Les cellules, plus particulièrement les fibroblastes ou les cellules cancéreuses, rampent et se propagent à la surface de ces membranes.

2. Un deuxième procédé, utilisé plus spécialement dans les cultures de tumeurs, consiste à interposer une membrane vitelline entre les explants d'organes embryonnaires et les explants de tumeurs. Ceux-ci, au lieu de se nourrir directement des cellules normales, sont alimentés d'une part par le milieu, d'autre part par des substances élaborées par les organes embryonnaires qui passent par dialyse

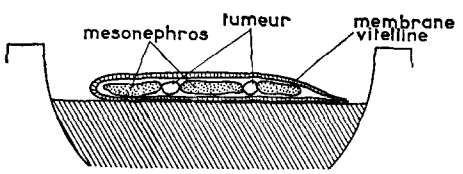

Fig. 2. Association d'explants de tumeur et de mésonéphros sur le milieu de culture. Les explants son enveloppés dans une membrane vitelline.

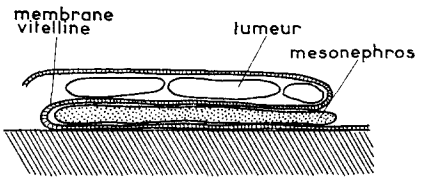

Fig. 3. Schéma, très simplifié, de la technique de la dialyse à travers la membrane vitelline de l'oeuf de poule. Cette membrane sépare les explants tumoraux de la couche du mésonéphros.

à travers la membrane. En pratique, on enveloppe respectivement chacun des deux types d'explants dans un pli de la membrane. Les fragments d'organes embryonnaires se trouvent dans un compartiment inférieur placé sur le milieu, les fragments de tumeurs à l'étage supérieur, séparés des organes embryonnaires par une épaisseur de membrane vitelline (Fig. 3).

On obtient ce résultat de la manière suivante. Pour la mise en culture des fragments d'organe (le mésonéphros par exemple), on procède comme dans le dispositif précédent. On a cependant eu soin de prendre un fragment de membrane vitelline de plus grande longueur, afin de pouvoir le replier deux fois sur lui-même. Les explants embryonnaires une fois recouverts de la membrane vitelline, on dispose les morceaux de tumeur sur la face supérieure de cette membrane. Puis on replie une deuxième fois la membrane, de manière qu'elle recouvre les explants tumoraux. Ainsi les deux sortes d'explants sont placés dans deux plis superposés de la membrane vitelline.

C'est grâce à ces techniques employant la membrane vitelline que nous avons réussi à cultiver des tumeurs organisées, provenant de souches cellulaires, ou de tumeurs humaines prélevées directement sur le patient [9, 10, 12].

II. CULTURES DE LONGUE DUREE DE TUMEURS ASSOCIEES DIRECTEMENT AU MESONEPHROS

Nous entretenons deux souches organoïdes depuis respectivement 4 ans et 2 ans 2 mois.

L'une d'elle, que nous appelons Z 200 [14, 17, 18] est issue d'une métastase hépatique d'un foyer primaire non reconnu, très vraisemblablement d'origine gastrique. La tumeur avait été prélevée, au cours d'une opération chirurgicale, sur un malade de sexe féminin âgé de 34 ans.* Cet adénocarcinome était caractérisé par des cordons épithélioïdes irréguliers, mêlés à un abondant stroma, entourant de place en place des cavités mal délimitées remplies de mucus.

Des fragments de cette tumeur ont été mis en culture le 10 janvier 1962, au contact d'explants de mésonéphros de poulet. Ils sont repiqués à intervalles réguliers, tous les 7 à 10 jours. Les explants tumoraux s'accroissent de 2 à 3 fois leur volume initial pendant ce laps de temps. Ils continuent à prospérer après 48 mois, correspondant à 168 repiquages.

A l'aspect extérieur, ils se présentent sous forme de nodules sphériques ou discoïdes, entourés d'une frange irrégulière qui correspond à la zone d'invasion des cellules tumorales dans le mésonéphros (Fig. 4a). Ces nodules ont un diamètre de 1 à 3 mm. A l'aspect histologique, ils ont une structure plus régulière que la tumeur initiale. Ils sont débarrassés du stroma conjonctif et sont constitués de tissus cancéreux purs. Ils forment des cordons épithélioïdes autour de cavités à mucus, arrondies ou étirées, à contours nets. L'ensemble évoque une glande excrétrice (Fig. 4b).

L'autre souche organoïde que nous avons appelée Z 516 [18] provient d'un épithélioma cylindrique du côlon, prélevé sur une femme de 80 ans, au cours d'une opération chirurgicale. Cette tumeur présentait une structure lâche, faite de cordons épithéliaux discontinus, entourés d'un stroma peu dense et dispersés dans une substance d'aspect muqueux.

Des fragments de cette tumeur ont été mis en culture le 29 Octobre 1963, associés à du mésonéphros d'embryons de poulet de 8 jours 1/2. Ils sont repiqués tous les 7 jours environ sur des milieux neufs contenant du mésonéphros frais. Ils survivent et se multiplient activement après plus de 26 mois†, ce qui correspond à 109 repiquages.

A l'aspect extérieur, ils se présentent comme des amas à contours irréguliers, mais assez distincts du mésonéphros. Leur surface est sillonnée de rides profondes et sinueuses (Fig. 5a). (Ce caractère s'accentuera encore dans les cultures sur membrane et dans les cultures sur levures). A l'aspect histologique, la tumeur a tendance à former des amas bien individualisés par rapport au mésonéphros et à proliférer par des expansions à bords nets et lobés. Des cordons épithéliaux limitent de grandes cavités à mucus (Fig. 5b). Ils sont généralement assez épais en surface, où leurs cellules forment une assise haute, ils sont grêles et étirés en profondeur.

*Les deux tumeurs nous ont été fournies par le Professeur L. LEGER, chirurgien de l'Hôpital Necker, que nous tenons à remercier de sa coopération amicale et efficace.

†En réalité, la culture directe sur mésonéphros a été abandonnée après 20 mois, et continuée uniquement sur membrane filtrante.

III. CULTURES SUR MESONEPHROS AVEC MEMBRANE INTERPOSÉE

A. *Membrane vitelline*

Grâce à ce deuxième procédé de culture, nous avons démontré que les tumeurs peuvent se nourrir autrement qu'en parasites des tissus normaux. On peut interposer entre ceux-ci et les explants tumoraux une membrane dialysante. Les nodules cancéreux utilisent les substances qui passent à travers la membrane, en même temps que les aliments du milieu. Ce second mode de culture est au moins aussi efficace que le premier. Nous le préférons actuellement au premier que nous avons abandonné, dans le cas de la tumeur Z 516. Nous entretenons les deux tumeurs suivant ce procédé, respectivement depuis 35 mois (Z 200) et 23 mois (Z 516) [20]. Très rapidement elles se purifient des cellules normales qui les accompagnaient. Ainsi, elles ne sont plus associées à aucune cellule humaine du stroma, à aucune cellule de l'embryon de poulet. Ce sont donc des cultures absolument pures de tissus cancéreux que l'on obtient dans ces conditions. Leur aspect morphologique et histologique devient alors très caractéristique et se révèle comme spécifique d'une tumeur donnée.

Ainsi, la tumeur Z 200 forme des amas à bords nets, à surface lisse, à contenu homogène (Fig. 6a). La tumeur Z 516 est remarquable par son aspect "cérébroïde". Ses bords sont irréguliers, sa surface est creusée de profonds sillons sinueux isolant des lobes et des lobules de substance tumorale (Fig. 6b).

A l'aspect histologique, les deux souches conservent en gros les caractères qu'elles avaient dans la tumeur initiale, mais ces caractères sont plus nets: la tumeur révèle sa morphologie à l'état pur et indépendant. Ainsi la tumeur Z 200 forme des massifs épithélioïdes creusés de cavités plus ou moins vastes, souvent aplaties parallèlement aux surfaces de l'explant, parfois étirées dans le sens transversal (Fig. 7a).

La tumeur Z 516 est constituée de cordons épithéliaux festonnés formant un "cortex" autour de grands amas de mucus. Ce cortex se replie sur lui-même et envoie dans la profondeur des cordons obtus qui tendent à cloisonner le mucus. Il se découpe souvent en nodules ou îlots isolés les uns des autres. (Fig. 7b).

B. *Membrane de cellophane*

Il est difficile d'évaluer, même approximativement, le diamètre des pores de la membrane vitelline. Nous savons, par différents recoupements, qu' elle ne laisse passer que des molécules relativement petites.

Pour pouvoir apprécier plus exactement les dimensions des molécules utilisées dans les expériences de dialyse, nous avons remplacé la membrane vitelline par des membranes de cellophane pour dialyse. Nous nous servons d'un dispositif analogue aux chambres de Rose, modifié dans notre laboratoire par P. Goube de Laforest [21, 22]. La chambre de culture est divisée en deux étages par une membrane de cellophane. Dans la chambre inférieure, on place le milieu de culture et le mésonéphros. Dans la chambre supérieure, on dispose les explants cancéreux sur la membrane de cellophane. Celle-ci est serrée par des écrous entre les montures des deux chambres (Fig. 8). On remarquera que la membrane filtre aussi bien les constituants du milieu que les substances élaborées par le mésonéphros.

Dans ces conditions, nous avons réussi à cultiver la tumeur Z 200 pendant 27 semaines, au cours de 27 repiquages. L'expérience continue. Les témoins sans mésonéphros n'ont jamais dépassé, dans les meilleurs cas, le 3e repiquage. En général, ils périclitent après 2 repiquages. Ainsi l'on peut affirmer que ce sont des substances de poids moléculaire inférieur à 14.000 qui favorisent la croissance des deux souches tumorales.

Ce résultat permet de tirer deux conclusions importantes.

1°— La tumeur peut utiliser de petites molécules fournies par le milieu de culture comme aliments essentiels. Ce sont vraisemblablement des peptides ou polypeptides peu complexes.‡

2°— Les substances de croissance issues du mésonéphros, qui filtrent à travers la membrane, sont aussi constituées de molécules de petites dimensions, de poids moléculaire inférieur à 14.000.

IV. CULTURES SUR EXTRAITS DE LEVURE

Des expériences qui viennent d'être relatées, il résulte que le mésonéphros agit sur la croissance des explants tumoraux par l'intermédiaire de substances diffusées, de faible poids moléculaire.

‡Ainsi qu'il résulte de recherches faites dans nos laboratoires sur l'utilisation de milieux synthétiques, les organes embryonnaires peuvent se cultiver exclusivement sur des mélanges plus ou moins complexes d'acides aminés [23].

Quelle est la nature, quelles sont les propriétés de ces substances? Il convenait de voir si des extraits de mésonéphros conserveraient l'activité de ce tissu vivant. Jusqu'à présent, les expériences n'ont pas donné de résultats positifs, soit que les substances actives ne soient élaborées que par un mésonéphros vivant, soit que les extraits soient toxiques pour les explants tumoraux.*

C'est pourquoi nous nous sommes adressés à d'autres extraits de cellules vivantes, riches en principes nutritifs et stimulateurs de croissance. Nous avons préparé des extraits délipidés de levures [24, 25].

A. *Préparation de l'extrait*

Nous prenons 100 g d'une levure "haute" de l'espèce *Saccharomyces cerevisiae*. Nous y ajoutons 1 l d'acétone. Nous broyons le mélange dans un microbroyeur, puis nous le centrifugeons à 10.000 g pendant 15 minutes. Le résidu est repris dans cinq fois sa masse de liquide de Tyrode, il est incubé pendant deux à trois heures à 37°, puis centrifugé à 10.000 g pendant 15 minutes. C'est le surnageant que nous incorporons aux milieux de culture, à raison d'un volume pour 19 volumes de milieu.

B. *Résultats*

Dans ces conditions, les explants des souches Z 200 et Z 516 survivent et s'accroissent très rapidement, malgré l'absence de tout substratum vivant. Nous avons utilisé, comme explants de départ, des nodules tumoraux cultivés depuis plusieurs mois sur une membrane vitelline les séparant des explants de mésonéphros. La croissance est considérable: les nodules augmentent de 2 à 4 fois leur volume en 7 jours. On peut multiplier le nombre des explants en les fragmentant à chaque transfert. Nous entretenons actuellement, sur extrait de levure, 690 explants de la tumeur Z 200 depuis 350 jours (49 repiquages), 280 explants de la tumeur Z 516 depuis 189 jours (26 repiquages).

Les témoins, cultivés sur les milieux sans extrait de levure, ne s'accroissent pas, ils dégénèrent entre le 1er et le 2e repiquage (7 à 14 jours).

1. *La souche* ♂ 200 — Les explants de cette tumeur acquièrent une morphologie très caractéristique, qui diffère considérablement, comme nous le verrons, de la tumeur Z 516. Ils ont l'aspect de sphérules ou d'ovoïdes aux bords francs, à la surface lisse, au contenu homogène et translucide (Fig. 9a).

Ces caractères s'esquissaient déjà dans les cultures sur membrane dialysante, ils s'accentuent dans les cultures sur extrait de levure, où les nodules cancéreux sont environnés de toutes parts par le milieu favorable, et ne s'attachent plus à une région localisée de la membrane vitelline, celle qui était superposée au mésonéphros.

La structure histologique de cette tumeur, tout en conservant dans l'ensemble ses caractères initiaux, se modifie cependant sensiblement. Les explants sont constitués d'amas lenticulaires de tissus denses, au milieu desquels se creusent des alvéoles, remplis de mucus, généralement moins nombreux et plus petits que dans les cultures sur mésonéphros (Fig. 9b). Autour de ces alvéoles, les cellules tumorales forment souvent des épithéliums réguliers, cylindriques ou cubiques. Dans les massifs pleins, les cellules sont polygonales et ont un aspect épithélioïde. La surface de ces massifs est souvent couverte par un épithélium cylindrique à cellules hautes, dont les noyaux allongés sont régulièrement disposés parallèlement aux membranes cellulaires et situés du côté basal. Les mitoses sont très nombreuses dans toute la masse des explants. La principale originalité de cette structure est due à ce que les explants sont constitués de tissus compacts, mieux ordonnés et moins sécréteurs, que dans les cultures sur mésonéphros.

2. *La souche* ♂ 516 — Contrairement aux nodules de la souche Z 200, ceux de la souche Z 516 ont un contour irrégulier, découpé par de profonds sillons. Leur surface supérieure reflète le même caractère. Elle présente de véritables "circonvolutions" qui accentuent son aspect cérébroïde (Fig. 10 a et b). De profondes scissures séparent de grands lobes, eux-mêmes découpés en lobules par des sillons secondaires. On peut se rendre compte, au seul aspect extérieur, que certaines de ces circonvolutions sont des îlots complètement détachés de la masse principale de la tumeur.

A l'aspect histologique, on voit des banderoles épithéliales, plus ou moins sinueuses, enveloppant l'ensemble du nodule comme un cortex (Fig. 11a). Elles envoient à l'intérieur du nodule des cordons épais, sinueux, et parfois ramifiés. La cavité du nodule, très spacieuse, est remplie de mucus. Parfois, les nodules baignent dans un abondant mucus, endigué par la membrane vitelline dont on les enveloppe, et qui favorise la culture. Ils se fragmentent souvent en cordons ou en massifs autonomes; ceux-ci essaiment de petits îlots de cellules qui constituent de nouvelles colonies

*Depuis que cet article a été donné à l'impression, nous avons réussi à cultiver les tumeurs sur des dialysats d'extraits de foie et de mésonéphros.

(Fig. 11 b et c). Il est rare que des massifs compacts s'édifient. La paroi des nodules est de nature épithéliale (Fig. 11d). Elle est généralement plus épaisse du côté supérieur, par où s'effectuent les échanges, que du côté inférieur en contact avec le milieu. Elle est constituée d'une ou plusieurs couches de cellules cylindriques, parfois disposées très régulièrement contre la membrane vitelline. Cette tendance des cordons à se disposer à la périphérie des nodules, à se plisser, à se fragmenter en îlots, à occuper la plus grande surface possible, décèle leur affinité pour l'oxygène et leur lutte contre l'asphyxie, qui résulterait de leur immersion dans l'abondant mucus.

Ainsi des tumeurs humaines, de type organoïde, cultivées en l'absence de tout tissu vivant, tendent à réaliser une morphologie externe et une structure spécifique, beaucoup plus nettement caractérisées que dans la tumeur primitive.

L'extrait de levure a le même rôle indispensable que le mésonéphros pour la croissance des tumeurs organisées *in vitro*.

3. *Quels sont les principes actifs de l'extrait de levure?* — Cette question revient à se demander quelles sont les substances capables d'entretenir la croissance des cancers en culture organotypique; elle peut contribuer à élucider les conditions qui permettent au cancer de se développer dans l'organisme humain. Elle est l'objet de recherches biochimiques qui se déroulent actuellement dans notre laboratoire. Cette question sera traitée plus en détail dans un prochain article. On peut, dès maintenant, en résumer les principaux résultats. Partant de l'extrait actif de la levure, nous l'avons, en collaboration avec Y. Croisille (résultats inédits), dialysé contre du liquide de Tyrode. Le dialysat s'est révélé très favorable à la culture de la tumeur Z 200 qui continue à proliférer après 32 repiquages (231 jours). Dans les mêmes conditions, la tumeur Z 516 survit et se multiplie depuis 19 repiquages (140 jours). Il s'agit donc de substances d'un poids moléculaire inférieur à 15.000. Ces résultats rejoignent et confirment ceux que nous avons relatés plus haut à propos des substances actives du mésonéphros.

Nous procédons actuellement au fractionnement du dialysat pour caractériser avec plus de précision la nature des substances actives.

Ainsi, qu'il s'agisse du mésonéphros ou de l'extrait de levure, les composés qui favorisent la croissance des explants tumoraux doivent être recherchés dans la fraction dialysable et parmi des composés à faible poids moléculaire.

CONCLUSIONS GENERALES

Nous avons réussi à cultiver, pendant de longues périodes, des nodules organisés de tumeurs malignes humaines, prélevées sur le patient au cours d'opérations chirurgicales. Ces explants conservent leur structure histologique, leurs propriétés physiologiques initiales. Ils se multiplient à un rythme rapide, formant toujours de nouveaux massifs semblables à ceux dont ils proviennent. Il est certain que ces explants, après un nombre considérable de repiquages, ne contiennent plus aucun des facteurs qui pouvaient les stimuler dans l'organisme humain. Les conditions qui leur sont fournies en culture suffisent évidemment à leurs besoins nutritifs et de croissance; il ne fait aucun doute que, sauf accident, ces souches organoïdes pourront être entretenues indéfiniment.

Le développement des recherches s'est fait en trois étapes. Dans une première étape, les explants tumoraux ont été associés à des explants de mésonéphros d'embryons de poulet, cultivés eux-mêmes sur un milieu nutritif: les cellules tumorales vivent en parasites des tissus embryonnaires. C'est l'étape du parasitisme. Mais ce parasitisme n'est pas obligatoire pour la réussite des cultures. On peut interposer une membrane filtrante entre le mésonéphros et la tumeur. Celle-ci se nourrit des substances dialysées à travers la membrane. Enfin, nous avons réussi à substituer au mésonéphros un extrait délipidé de levure de bière qui peut, lui aussi, être remplacé par son dialysat. Des recherches sont en cours pour fractionner ce liquide et analyser les substances actives. C'est l'étape du fractionnement. Dès maintenant, l'on peut affirmer que ces substances ont un poids moléculaire faible, inférieur à 14.000. Les recherches se poursuivent dans deux directions: d'une part l'analyse de l'extrait de levure par fractionnements successifs du dialysat; d'autre part, l'addition, au milieu de culture, de constituants connus de l'extrait de levure, tels que vitamines et substances de faible poids moléculaire de différentes catégories.

Nos recherches démontrent que deux catégories de substances sont nécessaires à la culture organotypique de cancers humains.

1. des facteurs nutritifs qu'ils trouvent dans le milieu de culture. Ceux-ci sont appropriés à la culture d'organes embryonnaires, mais ils

ne suffisent pas, à entretenir la vie et la croissance des tumeurs.

2. des facteurs de croissance qu'ils trouvent soit dans le mésonéphros vivant de l'embryon de poulet, soit dans un extrait de levure, soit dans le dialysat de l'un ou l'autre de ces matériaux.

Ces deux catégories de facteurs sont vraisemblablement responsables des deux propriétés fondamentales des souches organoïdes de tumeurs: le maintien de l'organisation, la croissance indéfinie des explants tumoraux. La conservation de la structure histologique, l'acquisition d'une morphologie spécifique, sont dues vraisemblablement aux propriétés physiques et chimiques du milieu, ainsi qu'il ressort de recherches antérieures de notre laboratoire [26] sur le rôle de différents substrats. La survie et la prolifération des cultures peuvent être attribuées aux facteurs nutritifs et de croissance du milieu; les uns et les autres sont en voie d'être élucidés par l'analyse des milieux naturels et l'élaboration de milieux synthétiques à partir de substances chimiquement définies.

RÉSUMÉ

1. *Deux tumeurs malignes humaines du tube digestif* (Z 200 *et* Z 516) *sont entretenues respectivement depuis* 48 *mois et* 26 *mois en cultures organotypiques. Elles continuent de proliférer activement en conservant leur structure massive et organisée et leur propriété de sécréter du mucus. Elles constituent ainsi des souches organoïdes capables de se multiplier indéfiniment.*

2. *Au cours des étapes successives de la culture, les explants tumoraux se débarrassent des cellules normales et se libèrent de tout rapport avec d'autres tissus. Ils constituent des nodules de cellules tumorales pures. Leur morphologie externe et leur structure histologique prennent un aspect spécifique beaucoup plus caractéristique que dans leur site initial, l'organisme humain. Les deux tumeurs diffèrent tant par leur aspect extérieur que par leur organisation interne.*

3. *Trois étapes successives ont été réalisées. Dans une première étape, les explants tumoraux, prélevés directement sur le malade, sont associés à des explants de mésonéphros d'embryons de poulet de* 8 *jours* 1/2, *cultivés eux-mêmes sur un milieu nutritif. Les cellules malignes vivent en parasites des tissus embryonnaires. Les explants tumoraux organisés ne peuvent survivre et se multiplier sur le seul milieu nutritif favorable aux cultures d'organes embryonnaires.*

4. *Une importante modification de la technique consiste à interposer entre le mésonéphros et les nodules tumoraux une membrane dialysante. La tumeur ne se nourrit plus en parasite des tissus vivants; elle utilise les substances, filtrées par la membrane, qui émanent d'une part du milieu, d'autre part du mésonéphros. Les unes peuvent être considérées comme des substances alimentaires, les autres comme des facteurs de croissance. Cette technique remplace avantageusement la culture directe sur les explants de mésonéphros.*

5. *Dans une troisième étape, on substitue aux substances élaborées par le mésonéphros un extrait délipidé de levure de bière. Celui-ci fournit aux explants tumoraux des facteurs de croissance qui ont la même activité que les substances élaborées par le mésonéphros vivant.*

6. *Des substances dialysées à travers la cellophane à partir du mésonéphros vivant ou de l'extrait de levure ont une activité de croissance approximativement équivalente à celle du tissu vivant ou de l'extrait de levure. Les facteurs de croissance des cultures de tumeurs organoïdes sont donc représentés par des substances de poids moléculaire relativement faible, inférieur à* 14.000. *Des recherches sont en cours pour analyser les fractions actives des dialysats.*

SUMMARY

1. *Two human malignant tumours of the digestive tract (adenocarcinoma of the stomach Z200 and epithelioma of the colon Z516) have been cultivated respectively for* 48 *months and* 26 *months in organotypic cultures. They continue to proliferate actively and to form solid nodules which preserve the initial structure and the physiological properties (secretion of mucus) of the primary tumour. Thus they represent organoïd strains which are capable of proliferating indefinitely.*

2. *During the successive transfers, the tumorous explants get rid of normal human cells and of any connections with other tissues. Their morphological aspect and histological structure become much more characteristic as in their primary site, viz. the human body. The two tumours differ from one another by their external as well as internal morphology.*

3. *Three successive methods were performed. During the first period of our research, tumorous explants, which were taken directly from the patient, were associated with explants of* $8\frac{1}{2}$*-day chick mesonephros cultivated themselves on a nutritive medium. Under these conditions malignant cells live as parasites in the embryonic tissues. Organized tumours cannot survive and proliferate* in vitro *in the medium which is convenient for embryonic organs, without being associated with organs such as explants of mesonephros.*

4. *An important technical modification consists in interposing a dialysing membrane between the mesonephros and the tumorous explants. The tumour does no longer feed as a parasite on the living tissues, it utilizes substances filtrating through the membrane which arise from the medium and the mesonephros. Some can be considered as nutrients, others as growth promoting substances. This technique replaces beneficially direct associations with mesonephros.*

5. *In a third stage, substances elaborated by the mesonephros were replaced by a delipidated yeast extract, which was able to supply the tumourous explants with growth promoting substances showing the same activity as the living mesonephros.*

6. *Substances dialyzing through cellophane membranes from the living mesonephros or the yeast extract have approximately the same growth activity as the living tissues or the crude yeast extract. These growth promoting factors correspond to substances of a low molecular weight, less than* 14,000. *Experiments are now dealing with the analysis of fractions of the dialyzates and their growth promoting activity.*

ZUSAMMENFASSUNG

1. *Zwei menschliche Krebstumoren des Verdauungsapparates (Z 200 und Z 516) leben in organotypischer Kultur resp. seit je* 48 *Monaten, und je* 26 *Monaten. Sie fahren fort aktiv zu wachsen und behalten ihre massive organisierte Struktur, sowie das Vermögen Schleim abzusondern. Sie stellen also organoïde Stämme dar, die imstande sind, sich ins unendliche zu vermehren.*

2. *Während der ersten Phasen der Kultur sterben die normalen Zellen der Explantate ab; somit befreien sich die letzteren von jedem Zusammenhang mit anderen Geweben. Sie stellen reine Gewebeknoten von Krebszellen dar. Ihre äussere Morphologie sowie ihre histologische Struktur haben ein ganz spezifisches Aussehen, welches viel charakteristischer ist als jenes, welches sie in ihrer ursprünglichen Lokalisation im menschlichen Organismus hatten. Die zwei Tumoren unterscheiden sich sowohl durch ihr äusserliches Ansehen als wie durch ihre innere Organisation.*

3. *Die Versuche wurden in drei Stufen durchgeführt. In einem ersten Stadium wurden die Krebsexplantate, welche direkt aus dem Patienten entnommen worden sind, mit kleinen Mesonephrosfragmenten assoziiert. Die letzeren werden selber auf einem nahrhaften Medium gezüchtet. Die Mesonephrosfragmente stammen aus einem* 8 1/2 *Tage alten Hühnerembryo. Die Krebszellen leben als Schmarotzer auf dem embryonalen Gewebe. Wenn die organisierten Krebsfragmente ohne Zusatz von Mesonephros direkt auf dem Medium kultiviert werden, welches vorteilhaft angepasst ist für normale embryonale Organkulturen, können sie nicht weiterleben und wachsen.*

4. *Eine wichtige Änderung der Methode besteht darin, dass eine dialysierende Membran zwischen Mesonephrosfragmenten und Tumorenknötchen ausgebreitet wird. Der Tumor ernährt sich dann nicht mehr als Parasit des lebenden Mesonephrosgewebes, sondern er absorbiert die Stoffe welche durch die Membran filtrieren und welche einerseits vom Medium, anderseits vom Mesonephros stammen. Diese Substanzen können die einen als Nährstoffe angesehen werden, die anderen als Wachstumsfaktoren. Diese Technik ersetzt vorteilhaft die direkte Kultur auf Mesonephrosexplantaten.*

5. *In einem dritten Stadium ersetzten wir die durch den Mesonephros ausgearbeiteten Stoffe durch ein delipidiertes Hefeextrakt. Dieses liefert den Tumorexplantaten Wachstumsfaktoren, welche dieselbe Wirkung haben wie die Stoffe die durch den lebenden Mesonephros abgesondert werden.*

6. *Die durch die Zellophanmembran dialysierende Stoffe, welche entweder aus dem lebendigen Mesonephros oder aus dem Hefeextrakt stammen, haben ungefähr die selbe wachstumsfördernde Wirkung als das lebende Gewebe oder das Hefeextrakt. Die Wachstumsfaktoren der organotypischen Tumorkulturen sind also Stoffe von verhältnismässig kleinem Molekulargewicht, kleiner als* 14 000. *Versuche sind eingeleitet, um die verschiedenen aktiven Fraktionen des Dialysats zu analysieren.*

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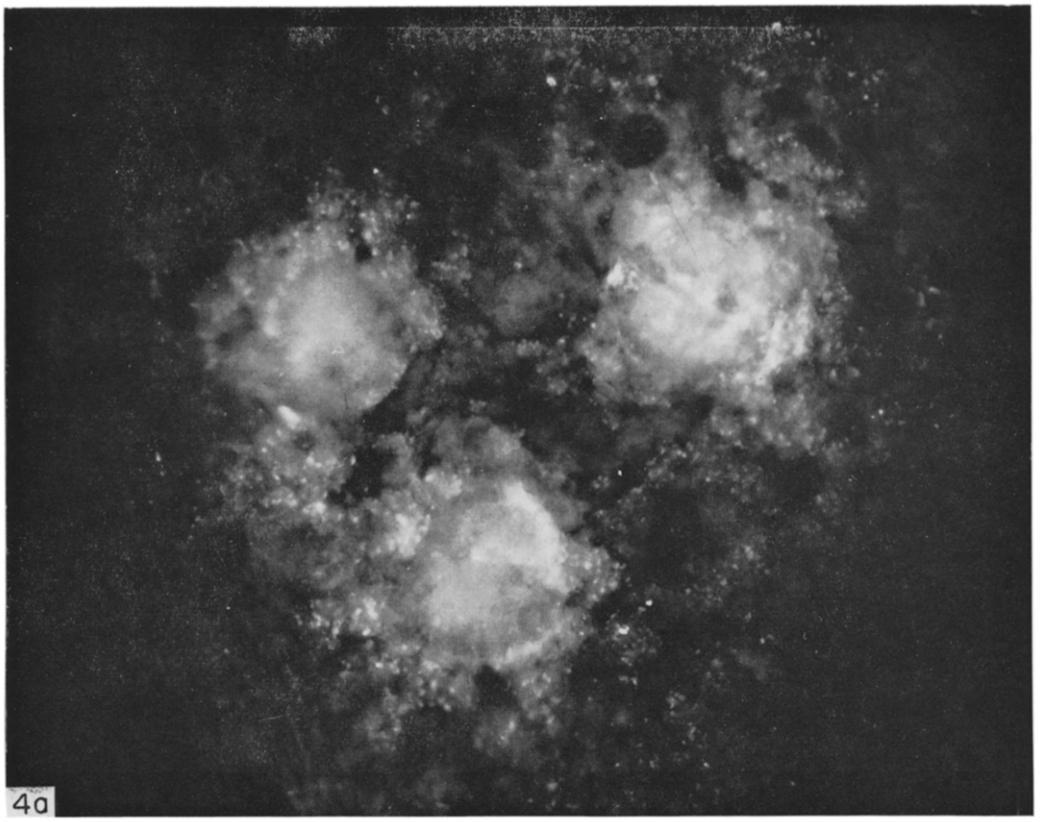

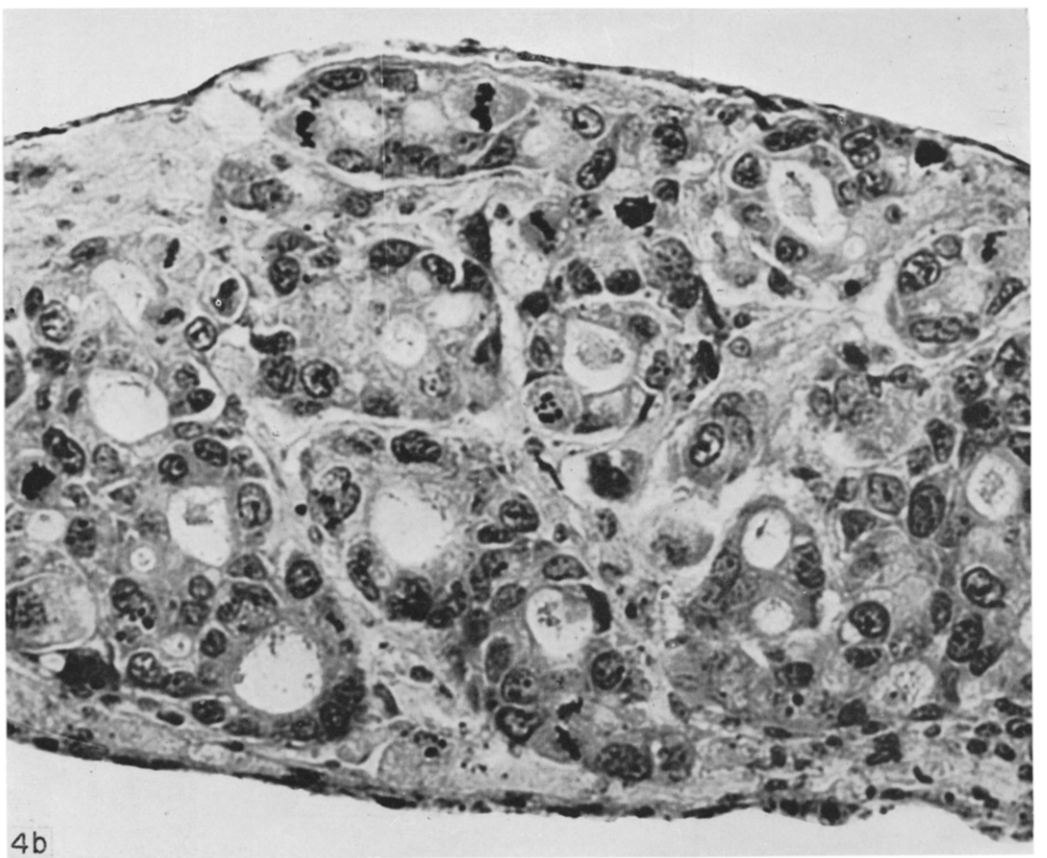

Fig. 4. *Culture de la tumeur digestive* Z 200 *sur du mésonéphros d'embryon de poulet.*

a. *Aspect macroscopique. Trois nodules tumoraux très clairs s'accroissent aux dépens du mésonéphros représenté par des explants granuleux plus sombres.* (34*e repiquage*) — *G. :* × 33.

b. *Coupe histologique à travers un nodule de la tumeur* Z 200 *cultivée sur mésonéphros. Cryptes à mucus, entourées de parois épithéliales.* (*Primoculture*). *G. :* × 450.

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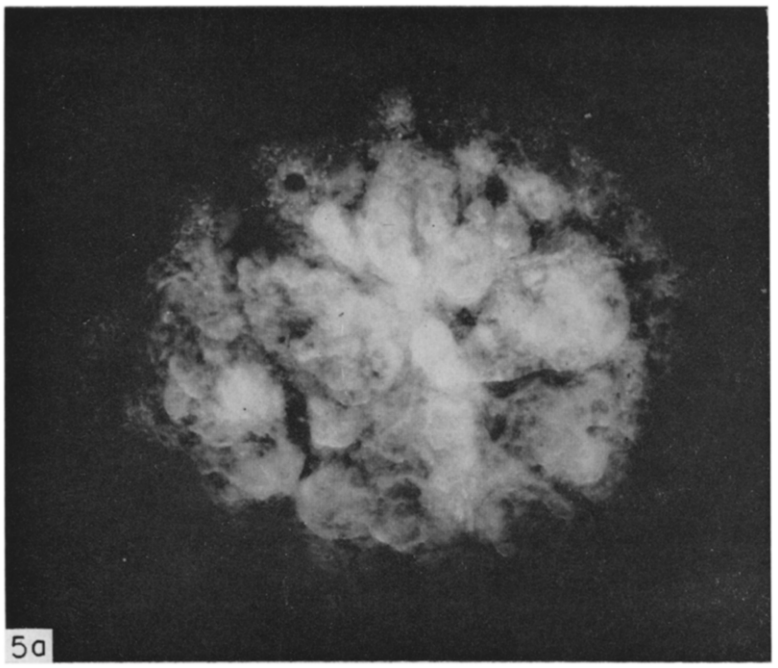


Fig. 5. *Culture sur mésonéphros de la tumeur* $\mathcal{Z}$ 516 (*épithélioma du côlon*).

a. *Aspect macroscopique. Les nodules tumoraux, découpés par de profonds sillons, envahissent la plus grande partie du mésonéphros, dont les bords sont encore visibles* (97*e repiquage*) — *G.* : × 20.

b. *Coupe histologique à travers les explants tumoraux qui s'immiscent entre les tubes du mésonéphros* (19*e repiquage*). *G.* : × 270.

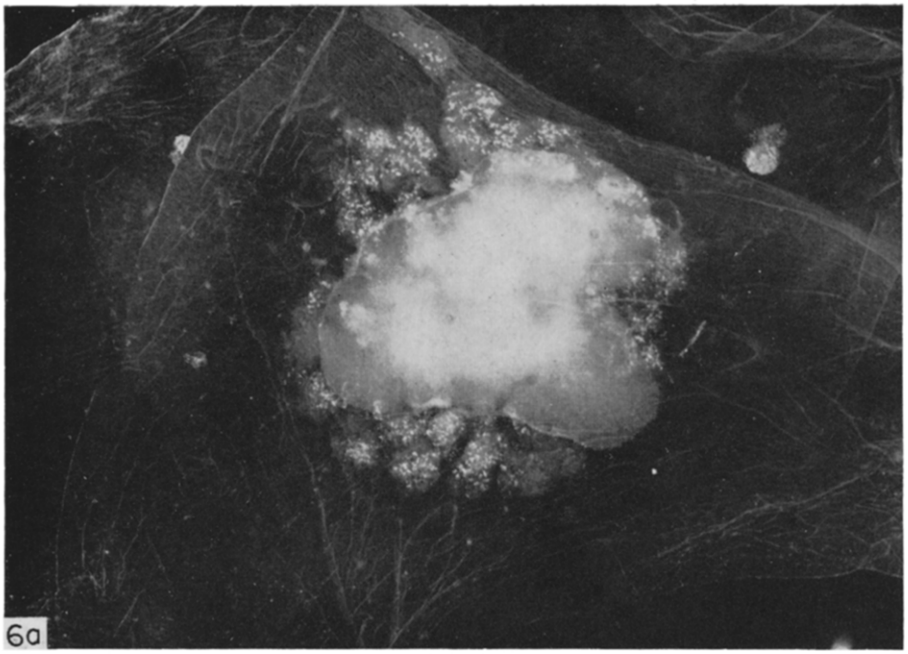


Fig. 6. *Cultures de tumeurs séparées du mésonéphros par une membrane filtrante.* (*Sur les trois photographies, on voit les plis de la membrane vitelline*).

a. *Culture de la tumeur* ♂ 200 *: Nodule à surface lisse, à bords nets. On voit, sur les bords de la tumeur, à travers la membrane vitelline, plusieurs massifs de mésonéphros* (154*e repiquage*). *G. :* × 15.

b *et* c. *Culture de la tumeur* ♂ 516. *Aspect "cérébroïde", avec des scissures profondes et des sillons séparant des îlots, des lobes et des lobules de substance tumorale. Le mésonéphros est complètement recouvert par la tumeur qui le déborde sur les côtés.* (96*e repiquage*). *G. :* × 15.

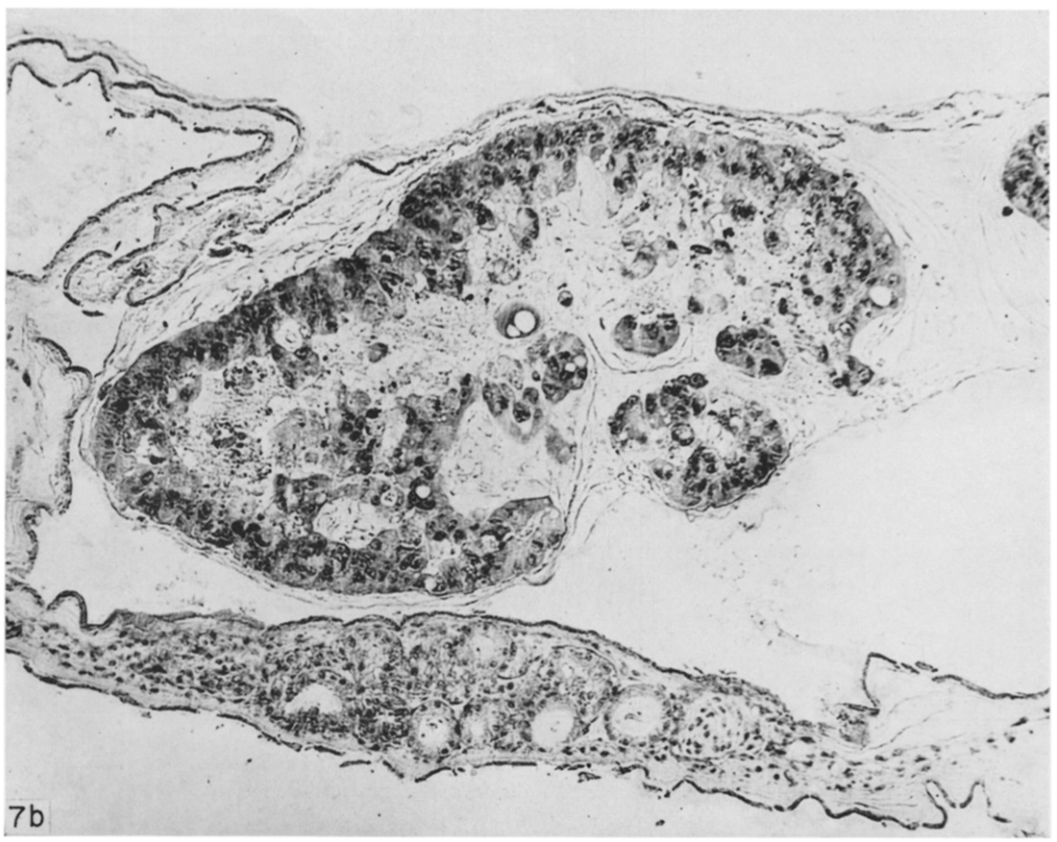
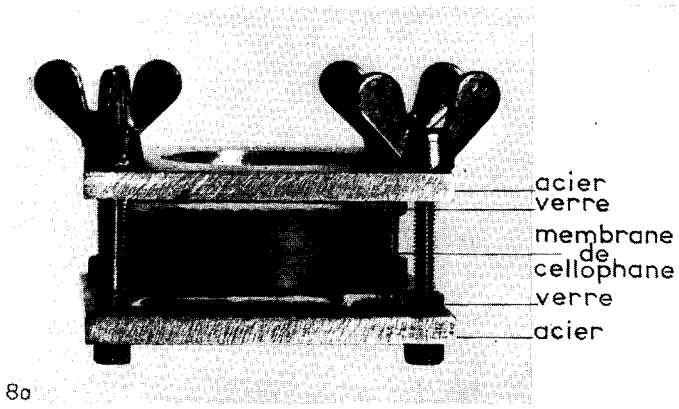


Fig. 7. Coupes histologiques à travers des tumeurs séparées du mésonéphros par une membrane filtrante.

a. *Souche organotypique ♂ 200 (75e repiquage dans ces conditions). Les deux étages d'explants sont séparés par la membrane vitelline. Celle-ci est repliée deux fois sur elle-même et enveloppe complètement les deux compartiments. (Voir schéma de la Fig. 3). G. :* × 200.

b. *Souche organotypique ♂ 516. (58e repiquage dans ces conditions). L'explant forme un cortex continu enveloppant du mucus et des îlots essaimés. Par un accident de fixation, la tumeur s'est considérablement décollée de la membrane dialysante qu'on voit intimement attachée à la couche du mésonéphros. G. :* × 168.



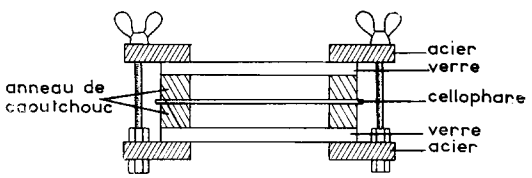


Fig. 8. Chambre de diffusion pour culture sur membrane dialysante.

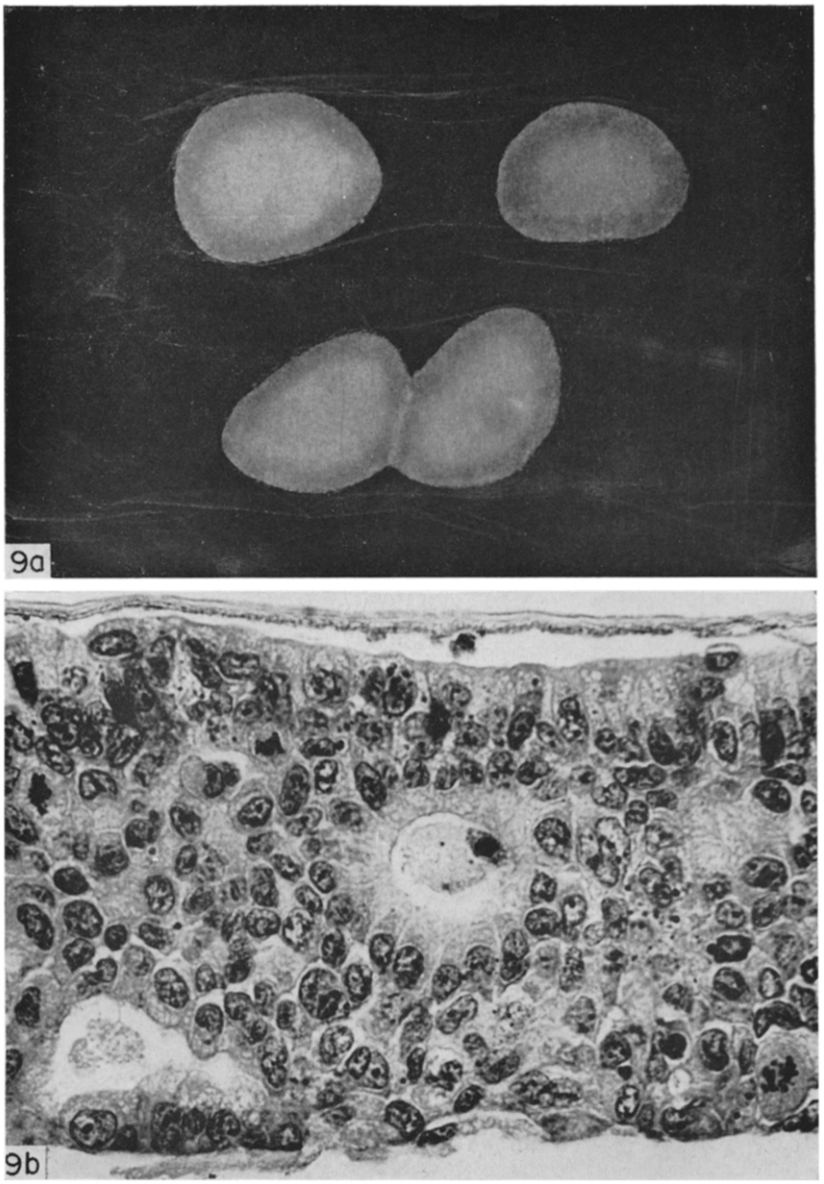


Fig. 9. *Cultures sur extrait délipidé de levure de la souche organotypique* Z 200.

a. *Aspect macroscopique de quatre nodules tumoraux, après* 23 *repiquages sur levure. G. :* × 15.

b. *Coupe d'un nodule cultivé dans ces conditions, après* 14 *repiquages. Noter l'aspect épithélial régulier de l'assise supérieure et des parois des cavités. Celles-ci sont moins nombreuses et en général plus petites que dans les cultures sur mésonéphros. G. :* × 420.

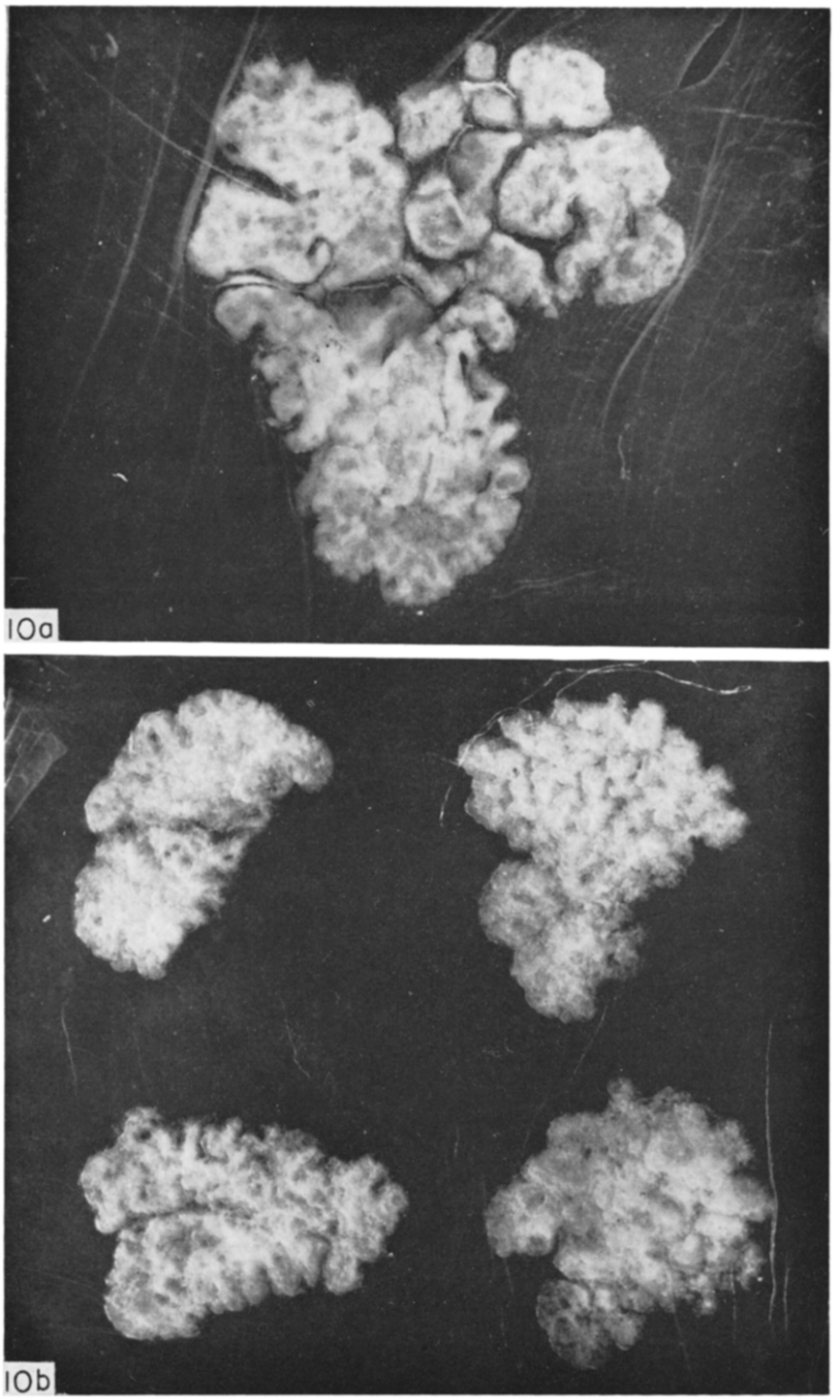


Fig. 10. *Cultures sur extrait de levure de la souche organoïde* $\mathcal{Z}$ 516. (25*e repiquage dans ces conditions*). *Noter l'aspect cérébroïde, tantôt profondément découpé, tantôt sillonné de fines échancrures, de ces explants dont la morphologie externe diffère considérablement de la tumeur* $\mathcal{Z}$ 200 (*comparer avec la Fig.* 9a).

a. *Trois nodules ont conflué en un seul explant.*

b. *Quatre nodules isolés, montrant d'innombrables sillons et lobules. G.:* × 15.

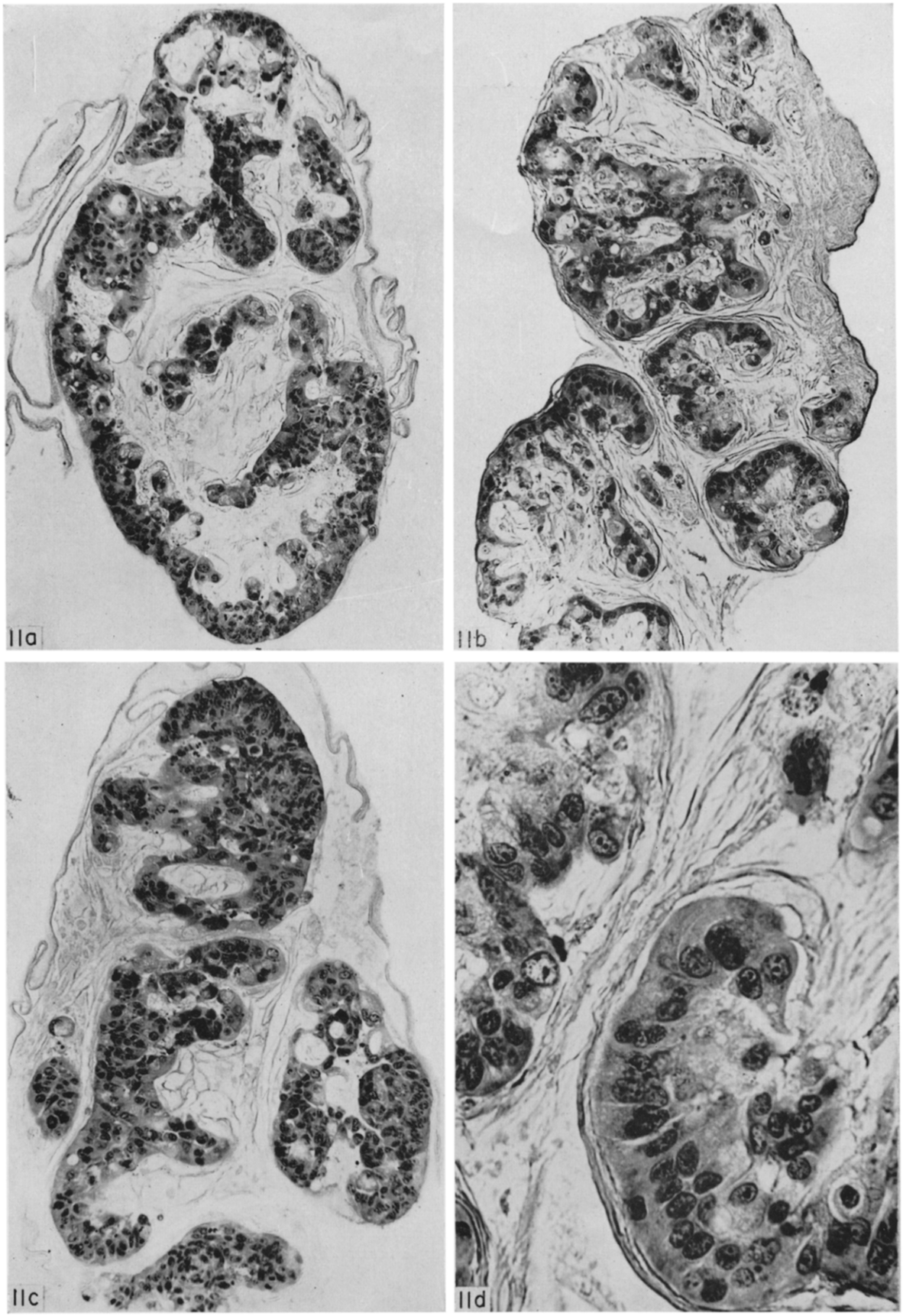


Fig. 11. a *et* c. *Aspect histologique des nodules de la souche organoïde* Z 516 *cultivés sur extrait de levure. (Après* 15 *repiquages dans ces conditions). Noter le cortex continu prolongé par de nombreuses travées intérieures* (a) *ou découpé en masses plus ou moins isolées* (b *et* c), *à structure épithéliale* (d). *G. :* a *et* b. *:* × 100, c. *:* × 130, d. *:* × 400.

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An Attempt to Study Cancer Dissemination*

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THE MALIGNANCY of tumours is mostly the result of the metastatic dissemination of cancer cells. This problem is difficult to study experimentally because most of the animal tumours used for cancer chemotherapy do not induce reproducible and measurable metastases.

Several authors in an effort to find a model for this type of investigation injected cancer cells intravenously [1–4]. This method, although having the advantage of being quantitatively controlled, is rather remote from the clinical situation because (a) there is not a primary tumour able to continuously supply cancer cells; (b) usually the number of cells injected is rather large; (c) normal animals are used instead of animals already depressed in their defences because of the tumour growth.

During a recent investigation concerning the growth of the uterine epithelioma (T_8) transplanted intracerebrally [5] it was noted that cancer cells were present in the blood. This observation represents the basis of the method described here, aiming at establishing a semiquantitative test to evaluate cancer dissemination.

MATERIALS AND METHODS

Sprague-Dawley rat of the average weight of 160 g $\pm$ 10 were used. The animals had free access to food (Diet Alal 56 of Alal Firm, Milano) and water, during the experiments. They were kept in cages of Makrolon (cm 47 $\times$ 26 $\times$ 15) six animals per cage, at constant temperature (22°) and relative humidity (60%).

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The technique used to transplant T_8 uterine epithelioma intracerebrally or subcutaneously has been described in previous papers [5, 6].

In order to detect the possible presence of neoplastic cells in the blood and in organs of rats bearing intracerebral or subcutaneously implanted T_8 tumour, blood clot or fragments of these organs were transplanted subcutaneously into normal rats.

The growth of a tumour in the recipient animal within 30 days from the transplantation was considered a proof that cancer cells were present in the blood or in the organs of the donor animals.

The blood was collected in sterile tubes after decapitation. The blood clot was cut into fragments of about 100–125 mm^3.

The organs for the transplantation were excised aseptically and cut in a watch glass into fragments weighing about 60–100 mg.

For the rats bearing intracerebral T_8 uterine tumours, lung, liver, kidney, spleen and blood clot were transplanted in addition to the brain. For the rats bearing subcutaneous T_8 tumours the axillary lymphnodes ipsilateral to the tumour and the abdominal lymphnodes were also transplanted.

Two or three fragments of each tissue were transplanted subcutaneously in the right retroscapular area of four different rats by means of a trocar. With the same technique only one piece of the blood clot was transplanted into every recipient rat. In every case the organs were transplanted within 30 min from the excision and during this time they were kept in saline at room temperature.

Table 1. *Incidence of subcutaneous tumour growth in recipient rats transplanted with tissues from rats bearing the T_8 tumour intracerebrally*

Days (°)	Number of subcutaneous tumour takes after transplantation of					
	Brain	Blood clot	Lung	Liver	Kidney	Spleen
2	4	2	—	1	—	—
4	3	3	—	—	—	—
6	4	—	1	—	—	—
7	2	2	—	1	—	—
8	4	3	3	2	2	3
9	4	—	—	1	—	—
10	3	1	2	—	—	—
11	3	3	4	1	—	—
12	4	4	1	2	—	—
13	4	3	—	—	—	—
14	4	4	4	—	2	—
Total	39/44	25/44	15/44	8/44	4/44	3/44

Each tissue has been transplanted in four rats at each time. (—)Failed to take.
(°) Time between T_8 tumour transplantation i.c. and s.c. transplantation of the tissues.

For the blood clot the transplantation took place 60 min after the blood collection. The tissues were excised at various times beginning from the second day after the i.c. or s.c. T_8 tumour transplantation.

RESULTS AND DISCUSSION

Table 1 and 2 report the results obtained in a typical experiment where blood clot or tissues obtained from animals bearing an intracerebral or a subcutaneous tumour (T_8 uterine epitelioma) were transplanted in normal rats. It can be noticed that the presence of cancer cells in blood is detected as early as 2 days after the intracerebral transplantation of the tumour (Table 1) while in the case of the subcutaneous tumour a period of more than 10 days is requested.

The times selected were related to the toxicity of the tumour for the rat. In fact the average survival time of rats implanted intracerebrally with T_8 tumour is about 14 days while about 50 per cent of the animals bearing a subcutaneous tumour are dead 26 days after the tumour transplantation.

The appearance of cancer cells in blood as well as in lung, liver and spleen seems to be more frequent when the tumour is transplanted intracerebrally than subcutaneously.

Table 3 summarizes the number of takes observed when similar tissues were obtained

Table 2. *Incidence of subcutaneous tumour growth in recipient rats transplanted with tissues from rats bearing the T_8 tumour subcutaneously*

Days (°)	Number of subcutaneous tumour takes after transplantation of					
	Axillary lymphnodes	Blood clot	Lung	Abdominal lymphnodes	Liver	Spleen
2	—	—	—	—	—	—
4	—	—	—	—	—	—
6	—	—	—	—	—	—
8	—	—	—	—	—	—
11	3	—	1	—	—	—
13	2	1	—	—	—	—
15	1	4	1	—	—	—
18	3	1	3	1	—	—
20	3	2	1	—	—	—
22	2	3	1	—	—	—
26	2	1	2	1	—	—
Total	16/44	12/44	9/44	2/44	0/44	0/44

Each tissue has been transplanted in four rats at each time. (—)Failed to take.
(°) Time between T_8 tumour subcutaneous transplantation and s.c. transplantation of tissues.

Table 3. Total number of tumour takes after subcutaneous transplantation of tissues excised from rats bearing intracerebral or subcutaneous T_8 uterine epithelioma

Tissue	Number of takes from tumour bearing animals	
	Subcutaneous	Intracerebral
Blood clot	12/44	25/44
Lung	9/44	15/44
Liver	0/44	8/44
Spleen	0/44	3/44
Total	21/176	51/176

from animals bearing intracerebral or subcutaneous tumour.

The fact that when the tumour is transplanted subcutaneously induces about the same frequency of cancer cells in blood and in lymphnodes would not support the assumption that T_8 tumour preferentially disseminates through the lymphatic system [7].

This method to evaluate cancer dissemination after transplantation of a tumour in the brain is currently used in this laboratory as a test for studies in cancer chemotherapy.

SUMMARY

Blood clot or tissues obtained from rats bearing an intracerebral or a subcutaneous T_8 uterine epithelioma contain cancer cells as demonstrated by the fact that they induce tumoural growth when implanted in other animals.

The cancer dissemination is more rapid and more frequent when the tumour is implanted intracerebrally than subcutaneously.

These observations will be applied to studies of cancer chemotherapy.

RÉSUMÉ

Du sang coagulé ou des tissus provenant de rats porteurs de l'épithélioma utérin T_8 inoculé par voie intracérébrale ou sous-cutanée, contiennent des cellules cancéreuses, comme le démontre la possibilité d'induire des tumeurs chez d'autres animaux, par l'implantation de ce matériel. La dissémination du cancer est plus rapide et plus fréquente lorsque la tumeur est implantée dans le cerveau que lorsqu'elle est inoculée dans le tissu sous-cutané. Ces observations seront appliquées à l'étude de la chimiothérapie du cancer.

ZUSAMMENFASSUNG

Blutgerinnsel oder Gewebe, von Ratten stammend, welche entweder mit einem intrazerebralen oder mit einem subkutanen T_8 — Gebärmutterepitheliom behaftet sind, enthalten Krebszellen, was aus der Tatsache hervorgeht, dass durch ihre Implantation in andere Tiere neue Tumorene entstehen.

Die Krebsaussaat geht schneller und häufiger vor sich, wenn der Tumor intrazerebral und nicht subkutan überpflanzt wird.

Diese Beobachtungen sollen bei Untersuchungen über die Chemotherapie des Krebses angewandt werden.

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Die Wirkung von Vincaleukoblastin (Velbe®) auf die Glykolyse von Tumorzellen*

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JEDE MITOSE benötigt Energie. Eine Störung des Energiestoff wechsels, z.B. ein Mangel an Energie, kann daher auch Rückwirkungen auf die Zellteilung haben. Alle onkolytisch wirksamen Alkaloide aus Vinca rosea blockieren bekanntlich *in vivo* und *in vitro* die Mitose in der Metaphase [1–7]. Es lag deshalb nahe zu prüfen, ob die durch Velbe induzierte Mitosehemmung die Folge einer Beeinträchtigung des Energiestoffwechsels sein könne, wie dies von Hunter [8] angenommen wurde. Einer der Lieferanten von energiereichen Phosphaten ist der Kohlehydratstoffwechsel. Um festzustellen, ob dieser durch Velbe beeinflußt wird, haben wir die anaerobe Glykolyse, die stationäre Konzentration des Coferments Nicotinamid-Adenin-Dinucleotid (NAD) und die Metabolite der Glykolyse Triosephosphate (TP) und 1,6-Fructosediphosphat (FDP) in Tumorzellen und proliferierenden Normalorganen (z. B. Darmschleimhaut) untersucht.

Ferner wurde die Ausscheidung an N^1-Methyl-Nicotinsäureamid (N^1-Methyl-NSA) im Urin von Ratten gemessen. Ihr Verhalten kann eine Störung im NAD-Stoffwechsel anzeigen. Erste experimentelle Ergebnisse wurden bereits mitgeteilt [9, 10].

*Herrn Prof. Dr. K. H. Bauer zu seinem 75. Geburtstag gewidmet.

METHODE

Die Bestimmung von NAD bzw. TP und FDP wurde nach Holzer u. Mitarb. [11] bzw. Thorn u. Mitarb. [12] ausgeführt. Bei der Messung der anaeroben Glykolyse folgten wir der Methode von Warburg. Sie wurde an Schnitten des Jensen-Sarkoms und in der Dünndarmschleimhaut jeweils zum Zeitpunkt der maximalen NAD-Erniedrigung durchgeführt. Die anaerobe Glykolyse der isolierten Yoshida-Ascites-Hepatom-Zellen (AH 130) wurde nach i.p. Applikation des Cytostaticums in den angegebenen Zeitintervallen ermittelt.

Im *in vitro*-Versuch erfolgte die Messung der anaeroben Glykolyse unmittelbar nach der Zugabe von Velbe.

Die Bestimmung des N^1-Methyl-NSA erfolgte in Anlehnung an die Methode von Carpenter u. Mitarb. [13]

Die i.p. Dosis von Velbe betrug 1,0 bzw. 0,5 mg/kg Körpergewicht. Die Konzentration an Cytostaticum *in vitro* wurde von 0,05 λ/ml auf 200 λ/ml gesteigert (Endkonzentration). Die Dosis von NSA, die gleichzeitig mit dem Cytostaticum i.p. verabreicht wurde, belief sich auf 100 mg/kg Körpergewicht. Die anaerobe Milchsäureproduktion wurde, wie üblich, als µl CO_2/Std. angegeben und ist beim Jensen-Sarkom auf mg Trockengewicht und beim AH 130 auf mg Protein bezogen.

Alle angegebenen Daten stellen die arithmetischen Mittelwerte der jeweils in Klammer angegebenen Anzahl von Einzelbeobachtungen dar.

ERGEBNISSE

Im Jensen-Sarkom ist 24 Std. nach i.p.-Injektion von 1,0 mg/kg Velbe eine Hemmung der anaeroben Glykolyse um 20% zu beobachten; sie beträgt in der Darmmucosa sogar 50% (Abb. 1 a und b). *In vitro*, an isolierten Yoshida-Ascites-Hepatom-Zellen ist eine Hemmung der

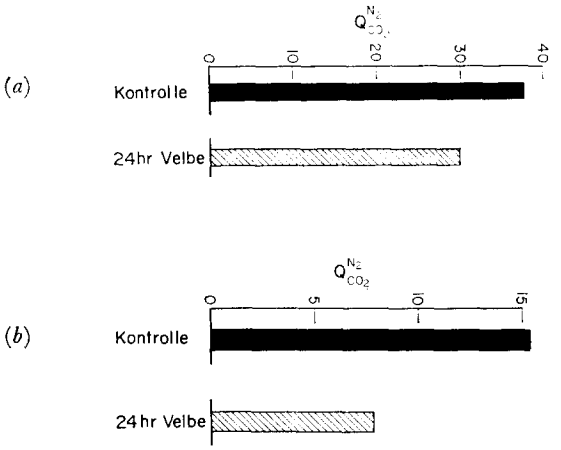

Abb. 1. *(a) Jensen-Sarkom der Ratte. (b) Dünndarmschleimhaut der Ratte. Anaerobe Glykolyse,* 24 *Stunden nach* 1,0 *mg/kg Velbe, i,p. Krebs-Ringer-Bicarbonat-Puffer:* $2 \times 10^{-2} M$ *NaHCO*$_3 + 1 \times 10^{-2} M$ *Glukose; pH* 7,4 *N*$_2$*/ QCO*$_2$ = µl CO_2*/mg Trockengewicht/h*

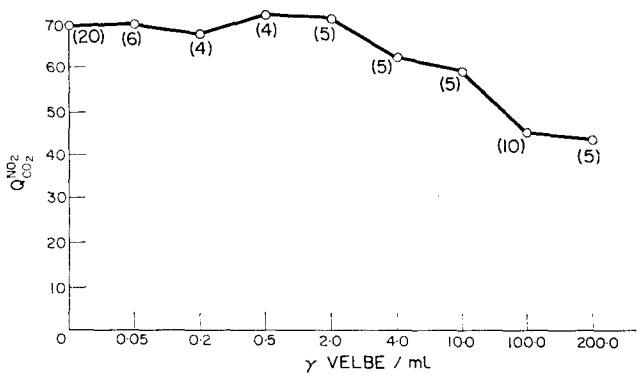

Abb. 2. *Anaerobe Glykolyse von Yoshida-Ascites-Hepatom-Zellen (AH* 130*) unter Einfluß von Velbe,* in vitro. *Krebs-Ringer-Bicarbonat-Puffer:* $2 \times 10^{-2} M$ *NaHCO*$_3 + 1 \times 10^{-2} M$ *Glukose; pH* 7,4*; ( ) = Anzahl der Tiere.*

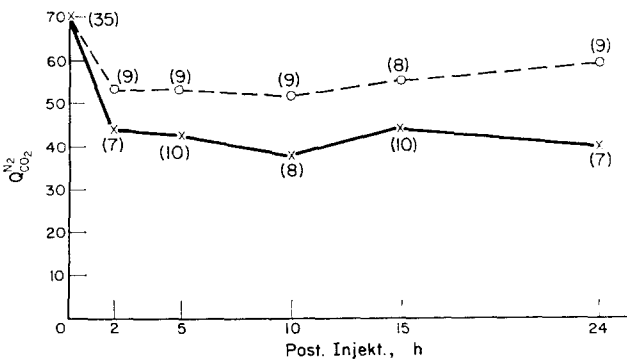

Abb. 3. *Anaerobe Glykolyse von Yoshida-Ascites-Hepatom-Zellen (AH* 130*) nach i.p. Injektion von Velbe. Krebs-Ringer-Bicarbonat-Puffer:* $2 \times 10^{-2} M$ *NaHCO*$_3 + 1 \times 10^{-2}$ *M Glukose; pH* 7,4. *( ) = Anzahl der Tiere;* ——— = 1,0 *mg/kg Körpergewicht;* – – – – – – = 0,5 *mg/kg Körpergewicht.*

anaeroben Glykolyse ebenfalls nachweisbar (Abb. 2). Sie tritt allerdings wie im Jensen-Sarkom nur bei hohen Dosen auf. Erst nach 100 γ/ml Velbe zeigt sich eine signifikante Erniedrigung um 40%. Ähnliche Ergebnisse finden wir beim gleichen Tumor nach i.p.-Injektion von Velbe (Abb. 3). Hier zeigt sich aber bereits unter der Dosis von 0,5 mg/kg (2 Std. nach einer Appl.) eine etwa 25%-ige Hemmung der anaeroben Glykolyse. Nach 1,0 mg/kg wird der Abfall mit maximal 45% (nach 10 Std.) noch deutlicher sichtbar. Es ist hinsichtlich NAD, TP und FDP im Prinzip dieselben Konzentrationsverschiebungen ab. Sie sind aber gegenüber den Veränderungen im Jensen-Sarkom stärker ausgeprägt. Wir beobachteten wieder einen schnellen Abfall des NAD um 50% und einen beträchtlichen Anstau der beiden Glucose-Metaboliten (Abb. 4b).

Ähnliche Untersuchungen am Yoshida-Ascites-Hepatom sind im Gange.

Um festzustellen, ob Velbe tatsächlich eine Störung des NAD-Stoffwechsels hervorruft, wurde die Exkretion an N¹-Methyl-NSA im

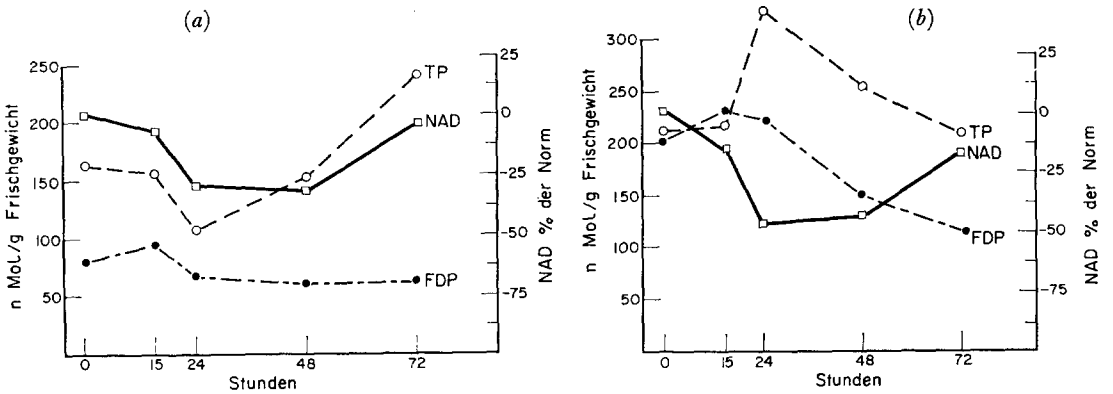

Abb. 4. *(a) Jensen-Sarkom; (b) Dünndarmschleimhaut der Ratte. Veränderungen der Konzentrationen an NAD, TP und FDP nach* 1,0 *mg/kg Velbe, i.p.*

bemerkenswert, daß die Glykolyse bei beiden Dosen sich nach 24 Std. noch nicht wieder normalisiert hat. Auch beim Jensen-Sarkom ist nach i.p. — Injektion von Velbe die verminderte Milchsäureproduktion nach 24 Std. noch nachweisbar.

Parallel zur Hemmung der Glykolyse findet im Jensen-Sarkom ebenso wie nach alkylierenden Cytostatica [14] ein Abfall der NAD-Konzentration statt, der nach 24 Std. bzw. 48 Std. sein Maximum mit etwa 30% erreicht (Abb. 4a). Dann kehrt das NAD schnell zur Norm zurück. Gleichzeitig kommt es zu einer Anhäufung der TP nach initialem, bisher noch ungeklärtem Abfall. Die FDP-Konzentration zeigt nur einen geringen Anstieg.

In der Dünndarmmucosa spielen sich Urin gemessen. Es ist bei der Ratte das Hauptausscheidungsprodukt von NSA [15]. Letzteres wird bekanntlich zur Biosynthese von NAD herangezogen [16–20]. Es zeigt sich, daß parallel zum Abfall der NAD-Konzentration schon 24 Stunden nach 1,3 mg/kg Velbe i.p. ein signifikanter Anstieg der N¹-Methyl-NSA-Ausscheidung auftritt. Diese nimmt an den folgenden Tagen noch weiter zu (Maximum: 135γ/24 Std.; normal : 72±12 γ/24 Std.). Danach fällt sie wieder ab. Werden die Tiere gleichzeitig einer oralen Belastung mit Tryptophan, der Ausgangssubstanz von NSA, unterworfen, so steigt die N¹-Methyl-NSA-Ausscheidung noch weiter bis auf das Vierfache des Kontrollwertes (377 γ/24 Std.) an (Abb. 5). Es ist noch darauf hinzuweisen, daß

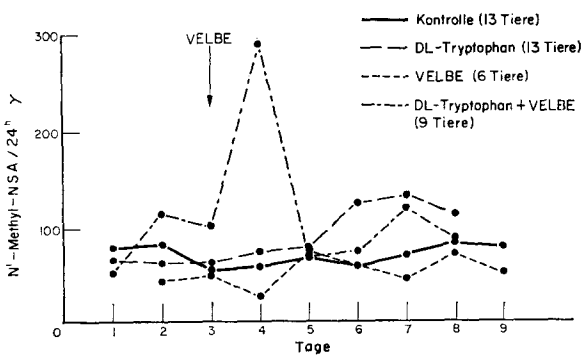

Abb. 5 *N¹—Methyl—NSA—Ausscheidung im Urinjensensarkomtragender Ratten nach* 1,3 *mg/kg Velbe i.p. und Tryptophan—Belastung* (230 *mg/kg p.o.*).

die gleichen Befunde, wie an jensensarkomtragenden, auch an normalen Ratten nach Velbe erhalten werden.

Als Metabolit von NAD stimuliert NSA *in vitro* die Biosynthese des Pyridinnucleotids [19]. Hohe Dosen von NSA vermögen sogar den durch Cytostatica ausgelösten Abfall von NAD

Das Coferment NAD steht in enger Beziehung zur Glykolyse, wo es der Phosphotriosedehydrogenase als Wasserstoff-Acceptor dient. Die "oxydierende Gärungsreaktion", wie man die phosphorylierende Dehydrierung von Glycerin-3-phosphat (GAP) in Anlehnung an Warburg auch nennt, wird allgemein, neben

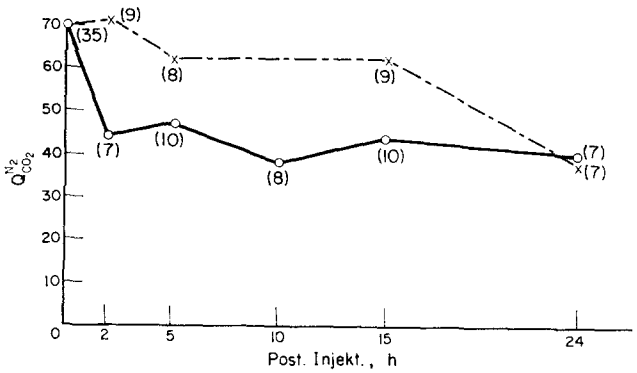

Abb. 6. Effekt von NSA (i.p.) auf die Hemmung der anaeroben Glykolyse von Yoshida-Ascites-Hepatom-Zellen (AH 130) nach Velbe. Krebs-Ringer-Bicarbonat-Puffer: 2×10^{-2} *M* $NaHCO_3 + 1 \times 10^{-2}$*M Glukose; pH 7,4.*
( ) = Anzahl der Tiere; ——— = 1,0 mg Velbe/kg Körpergewicht i. .—.—.— = 1,0 mg VELBE/Kg Körpergewicht i.p + 100 mg NSA/kg Körpergewicht i.p.

zu verhindern und den Hemmeffekt auf den Tumor aufzuheben [14].

Nach unseren Untersuchungen ist NSA (100 mg/kg i.p.) ebenfalls in der Lage, die Beeinträchtigung der Glykolyse in Yoshida-Ascites-Hepatom-Zellen nach Velbe vorübergehend zu verhindern (Abb. 6).

Nach 24 Std. hat die Konzentration von NSA im Ascites durch Resorption und Ausscheidung, Verbrauch und Verteilung wieder soweit abgenommen, daß der Schutzeffekt auf die Hemmung der Glykolyse nachläßt.

DISKUSSION

Die geschilderten Befunde zeigen, dass Velbe® die Glykolyse von Tumorzellen und Dünndarmschleimhaut trotz unterschiedlicher Konstitution wie alkylierende Zytostatika [21–23] zu hemmen vermag. Die Ursache für die Beeinträchtigung der Gärung ist für letztere nach Roitt [24] und Holzer [25] sowie eigenen Versuchen [10] in einer Erniedrigung des NAD-Spiegels zu suchen. Eine solche konnte nunmehr erstmals auch für Velbe nachgewiesen werden. Die Möglichkeit, über eine Stimulierung der Synthese dieses Pyridinnukleotids [19] durch NSA die Depression der Glykolyse wieder aufzuheben, erhärtet auch im Falle von Velbe seine ursächliche Bedeutung für die verminderte Milchsäurebildung.

der Hexokinasereaktion, als limitierender Schritt für den glykolytischen Abbau angesehen. Die Erniedrigung des NAD-Spiegels muß daher eine Verlangsamung der oxydierenden Gärungsreaktion zur Folge haben. Damit ist ein Anstau der Metabolite oberhalb dieses Schrittes verbunden. Wir haben denselben (Anstieg von TP und FDP) gleichfalls gefunden.

Die Senkung der NAD-Konzentration in den untersuchten Geweben kommt wahrscheinlich durch eine Störung der NAD-Synthese zustande. Dafür spricht der Anstieg des NSA nach Tetramin in Ascites-Tumorzellen, den Kröger u. Mit. [26] feststellen konnten. Die Zunahme der N^1-Methyl-NSA-Ausscheidung nach Velbe und Tryptophan in unseren Experimenten läßt sich wahrscheinlich in gleichem Sinne deuten, zumal diese nach alleiniger Tryptophangabe nur wenig ausgeprägt ist. Andererseits spricht aber gerade das Verhalten der N^1-Methyl-NSA-Exkretion nach Zytostatika und Tryptophan gegen einen vermehrten Abbau von NAD [27], da ein relativ rascher Austausch von NSA zwischen Milieu und Zellen erfolgt [19, 28, 29] und dieses die NAD-Nucleotidase hemmt [30, 31]. Zusätzlich aus dem verfütterten Tryptophan gebildetes NSA dürfte daher die von Hilz u. Mit. [27] vermutete "Enthemmung" der NAD-ase eher wieder aufheben und

zu einer Ausscheidungsrate von N^1-Methyl-NSA führen, die der nach Tryptophangabe (ohne Zytostatika) entspricht, das ist aber, wie wir gesehen haben, nicht der Fall. Als weiteres Argument gegen die Hypothese von Hilz u. Mit. [27] ist der bisher *in vitro* stets mißlungene Nachweis einer Aktivierung NAD-abbauender Enzyme anzuführen [32–34]. *In vivo* scheint jedoch eine Stimulierung der NAD-ase nach N-Lost unter gewissen Umständen möglich zu sein [35].

Die untersuchten Stoffwechselgrößen scheinen aber gegenüber Velbe weniger empfindlich zu sein als gegenüber den alkylierenden Substanzen [10]. Lediglich Hunter [8] berichtet über die Möglichkeit, *in vitro* schon mit 50 ppm (=50 γ/ml) Velbe die anaerobe Glykolyse von thioguaninresistenten L 1210-Leukämiezellen um 90% zu hemmen, während Johnson [36] mit Dosen von 15 und 150 γ/ml diese in Homogenaten von S-180-Ascites-Zellen nicht verändern konnte.

Die beobachteten Störungen traten bei den Tumorzellen, die wir verwendeten (Jensen-Sarkom und Yoshida-Ascites-Hepatom-AH 130) ebenfalls erst bei vergleichsweise hohen Dosen von Velbe (DL_{50}) auf. Cytotoxische Veränderungen *in vitro* sind demgegenüber schon nach so geringen Konzentrationen wie 0,002 γ/ml [6] nachzuweisen. Es erscheint daher unwahrscheinlich, daß die Glykolysehemmung nach Velbe als Ursache für die Tumorheilung oder die für diese Substanz typischen Mitosestörungen zu betrachten ist, so interessant und wichtig dieser Befund zur Aufklärung des Wirkungsmechanismus von Velbe auch sein mag.

Ob die Beeinträchtigung des NS-Stoffwechsels mehr im Vordergrund der Velbe-Wirkung steht, wie dies auf Grund der Untersuchungen Richards u. Mitarb. [37] sowie von Creasey [38] angenommen werden kann, ist noch nicht entschieden. Letztere erzielten eine 60%-ige Abnahme der H^3-Uridin-Absorption in die soluble RNA von Ehrlich-Ascites-Zellen, jedoch erst nach der Verabreichung einer Dosis von 2 mg/kg.

Anerkennungen — Diese Arbeit wurde mit dankenswerter Unterstützung des Badischen Landesverbandes zur Bekämpfung des Krebses und der Deutschen Forschungsgemeinschaft durchgeführt. Wir danken Frl. H. Miessner für ihre gute technische Hilfe.

Für die freundliche Ueberlassung der beiden Tumorstämme sind wir dem Direktor des Institutes für Präventivmedizin, Herrn Prof. Dr. H. Druckrey Freiburg und Herrn Doz. Dr. Oehlert aus dem Pathologischen Institut Freiburg zu Dank verpflichtet.

RÉSUMÉ

Les auteurs présentent un rapport concernant les altérations biochimiques de la glycolyse dans des cellules tumorales (sarcome de Jensen; hépatome ascitogène de Yoshida-AH 130) *et dans des cellules provenant d'un organe à prolifération rapide (muqueuse jéjunale).*

La vinblastine provoque in vivo *et* in vitro*—probablement par un ralentissement de la biosynthèse de NAD—un freinage de la glycolyse anaérobie qui peut être temporairement annulé par l'amide de l'acide nicotinique. Or, cet effet ne se fait sentir qu'après l'administration de doses relativement élevées de vinblastine, et ne devrait donc pas être décisif en ce qui concerne la disparition des tumeurs et le blocage des métaphases.*

SUMMARY

The authors present a report on the biochemical alterations of glycolyse in tumoural cells (Jensen's sarcoma; Yoshida ascitigenous hepatoma-AH 130) *and in cells originating in an organ with rapid cell proliferation (jejunal mucous membrane).*

Vinblastin causes a decrease in vivo *and* in vitro *of the anaerobic glycolyse—probably via a slower biosynthesis of NAD— which can temporarily be abolished by nicotinamide. However, this effect is only experienced after the administration of rather high doses of vinblastin and therefore should not be decisive as to the disappearance of tumours and the blockage of the metaphases.*

ZUSAMMENFASSUNG

Es wird über biochemische Veränderungen der Glykolyse von Tumorzellen (Jensen-Sarkom und Yoshida-Ascites-Hepatom-AH 130) *sowie von Zellen eines schnell proliferierenden Normalorgans (Dünndarmschleimhaut) berichtet. Velbe verursacht* in vitro *und* in vivo *wahrscheinlich über eine Hemmung der Biosynthese von NAD, eine Hemmung der anaeroben Glykolyse, die durch NSA vorübergehend aufgehoben werden kann. Sie tritt aber erst nach relativ hohen Velbe-Dosen auf und dürfte daher für die Tumorheilung und die Blockierung der Metaphasen nicht entscheidend sein.*

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Effects of Alkylating Agents on Thymidine Uptake by Mouse Thymocytes with Reference to an *in vitro* Sensitivity Test for Individual Human Tumours

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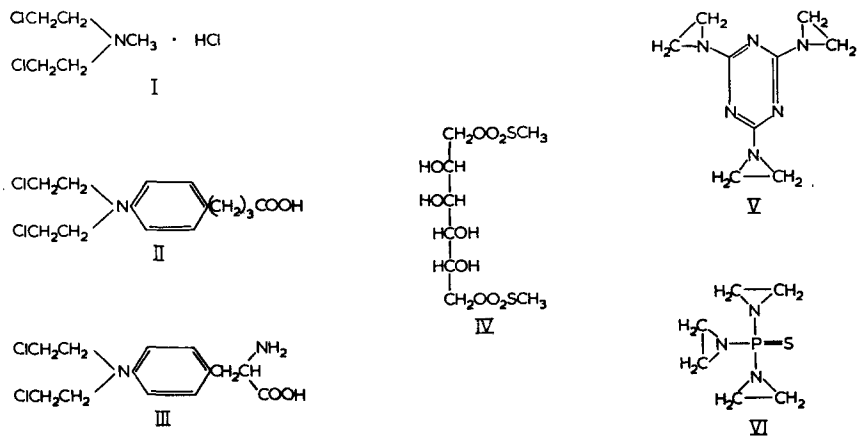
INTRODUCTION

IN RECENT years, there have been many attempts to find an *in vitro* method of determining the *in vivo* sensitivity of a given tumour in a given patient to various chemotherapeutic agents. Most of the proposed methods involve the incubation of small pieces of the tumour (or effusions) with various drugs, followed by an estimation of cell damage. The assumption is made that the drug most effective in damaging the tumour piece *in vitro* would be the most effective against the tumour in the patient, due allowance first being made for differences in drug toxicities. Damage may be assessed from changes in cell counts during culture [1], from cytological changes [2], or from biochemical analyses on the whole cell population with determination of protein content [3], dehydrogenase activity [4], or oxygen consumption [5]. Drug action on individual cells can be estimated from the two former methods but tissue culture over several days is usually involved and selection of the most resistant cell type may occur. Major disadvantages of the biochemical methods are that normal or necrotic areas within the tumour piece influence results and variations from cell to cell cannot be measured.

Wolberg and Curreri [6] and Wolberg and Brown [7] studied the effect of cancer chemotherapeutic agents on nucleic acid synthesis in slices of biopsied tumours. By following the uptake of tritiated thymidine or uridine autoradiographically, they obtained information on growth inhibition at the cellular level. Each tumour slice was incubated in tissue culture medium at 37°C with one of the drugs for $\frac{1}{2}$ or 1 hr. One of the labelled nucleosides was then added and incubation continued for a further $17\frac{1}{2}$ or 23 hr. The tissue was fixed, sectioned and exposed to autoradiographic stripping film. The effect of each drug on labelling of DNA and RNA was determined from grain counts. Similar work [8] was carried out on Ehrlich ascites cells exposed to drugs for 24 hr and to tritiated thymidine for a further hour. The advantage of examining uptake of labelled metabolites autoradiographically is that the response of individual cells, both neoplastic and normal, can be determined. These workers did not investigate in detail the effect of varying the time of preincubation with the drug. The following work shows that the apparent effectiveness of a drug depends on the time it acts on the cells and that this time-effect varies from one drug to another.

The importance of the incubation times in such an *in vitro* test was investigated on a standard cell system. Mouse thymus was chosen as this standard tissue because it consists of few cell types and contains cells with a high mitotic index.



Structures I-VI

MATERIALS AND METHOD

Mouse thymocytes

Female A2G mice, approximately 30 days old and weighing 9·3 – 15·6 g were obtained from Glaxo Laboratories, Harefield. A separate mouse was used for each drug assay. The mouse was killed with anaesthetic ether and its thymus aseptically removed into approximately 15 ml tissue culture medium. The thymus was teased into a cell suspension which was filtered through gauze to remove cell clumps. The cell concentration was adjusted to 3×10^6 cells / ml.

Tissue culture medium

This was Hanks' balanced salt solution enriched with 12·5% pooled A2G mouse serum which had been previously heated at 56°C for 30 min. Penicillin and streptomycin were added to give a final concentration of approximately 100 and 75 Units respectively per ml.

Drugs

Nitrogen mustard (I), di-2-chloroethylmethylamine hydrochloride, "Mustine Hydrochloride" (Boots). Molecular weight 192·5. Batch number 324W.

Chlorambucil (II), *p*-(di-2-chloroethylamino) phenylbutyric acid, kindly supplied by Dr. D. A. G. Galton, Chester Beatty Research Institute, London. Molecular weight 304·2. Batch number 1389.

Melphalan (III), L-*p*-(di-2-chloroethylamino)phenylalanine, "Alkeran" (Burroughs Wellcome). Molecular weight 305·2. Batch number 50993.

Mannitol myleran (IV), 1,6-(dimethanesulphonyl)-1,6-dideoxymannitol, kindly supplied by Dr. G. M. Timmis, Chester Beatty Research Institute, London. Molecular weight 338.

Tretamine (V), triethylenemelamine, (ICI). Molecular weight 204·2. Batch number UP272.

ThioTEPA (VI), triethylenethiophosphortriamide, (Lederle). Molecular weight 189·22. Batch number 4650-137A.

Drug diluents

Pyrogen-free distilled water was used for nitrogen mustard, thioTEPA, tretamine and mannitol myleran. Chlorambucil and melphalan, being insoluble in water, were dissolved according to the manufacturer's instructions in ethyl alcohol and acid-alcohol respectively and diluted with propylene glycol diluent as supplied by the manufacturer. All the tubes in the chlorambucil and melphalan series were adjusted to contain the same alcohol (or acid-alcohol) and propylene glycol concentrations. To neutralize the acid, 0·05 ml 5% sodium bicarbonate was added to all tubes in the melphalan series.

Tritiated thymidine

Tritiated thymidine-6-T(n), batch 30, with a specific activity of 8·8c / mM was obtained from the Radiochemical Centre, Amersham. At the appropriate time, 0·1ml of thymidine solution containing 1 μc / ml was added to each tube to give a final concentration of 0·1 μc / 3×10^6 cells.

Assay procedure

For each assay, 1-ml aliquots of cell suspension were placed in fifteen 16 × 150 mm pyrex test tubes, which were divided into three groups of five. Four dilutions of each drug were made immediately before use and 0·1 ml

of each solution added to the appropriate tubes. One-tenth of a millilitre of drug diluent was added to the three remaining tubes as controls. Thymidine was added to the first group of tubes immediately after the drug, to the second group after the cells had been incubated at 37°C with the drug for 45 min and to the third group after the cells had been incubated with the drug for 1½ hr. Tubes were incubated for 35 min after the thymidine had been added.

The cells were then centrifuged at 4°C and 650 g for 7½ min. The cell deposit was washed twice in 5% bovine plasma albumin (Armour) in saline, and the final deposit in each tube was smeared on fat-free slides previously treated with a solution of 0·5% gelatin and 0·1% chrome alum. All smears were fixed in methanol overnight. It was assumed that all detected radioactivity was confined to freshly synthesized DNA, since fixation in absolute methanol for as short a time as 3 min is reported to remove all precursor pool tritium [9].

Autoradiographic procedure

Kodak A.R. 10 stripping film was left standing over a saturated calcium nitrate solution at 4°C before it was applied to the slides [10]. The autoradiographs were exposed at 4°C for 1 or 2 weeks before being processed with D19B developer and Fix-sol (Johnson). These exposure times were chosen for ease of counting. It was found that the percentage cells labelled was most easily counted on smears exposed for 2 weeks but, since this exposure time resulted in some labelled cells showing grains too numerous to be counted with accuracy, the mean number of grains per labelled cell was determined from smears exposed for the shorter time. These two batches of autoradiographs were given random code numbers before independent examination by two observers. Sixty labelled cells were examined on each autoradiograph exposed for 1 week to give a mean number of grains per labelled cell, and a total of 3000 cells was examined on each autoradiograph exposed for 2 weeks to give a mean value for the percentage cells labelled.

RESULTS

Grains per labelled cell (*Figs.* 1–6)

Five of the six alkylating agents examined reduced the mean number of grains over labelled cells. These agents were nitrogen mustard, tretamine, melphalan, thioTEPA and chlorambucil. There were wide differences in the drug concentrations needed to decrease the average grain count below the control level. For example, with 1½ hr pretreatment, 5μg nitrogen mustard (0·02mM) had the same effect on thymidine incorporation as 1 mg thioTEPA (4·40mM). Up to 4·9mM mannitol myleran had no effect on the grain count.

Percentage cells labelled (*Figs.* 7–12)

With the exception of mannitol myleran, all alkylating agents examined reduced the number of cells synthesizing DNA. Up to 4·9mM mannitol myleran had no effect on the number of labelled cells. In general, response curves showing the effect of the drugs on the percentage cells labelled were similar to the corresponding curves showing the drug effect on the mean number of grains over labelled cells. The curves for nitrogen mustard are an exception to this, in that, at low drug concentrations and with no pretreatment, the number of grains per labelled cell was reduced to a greater extent than the percentage cells labelled.

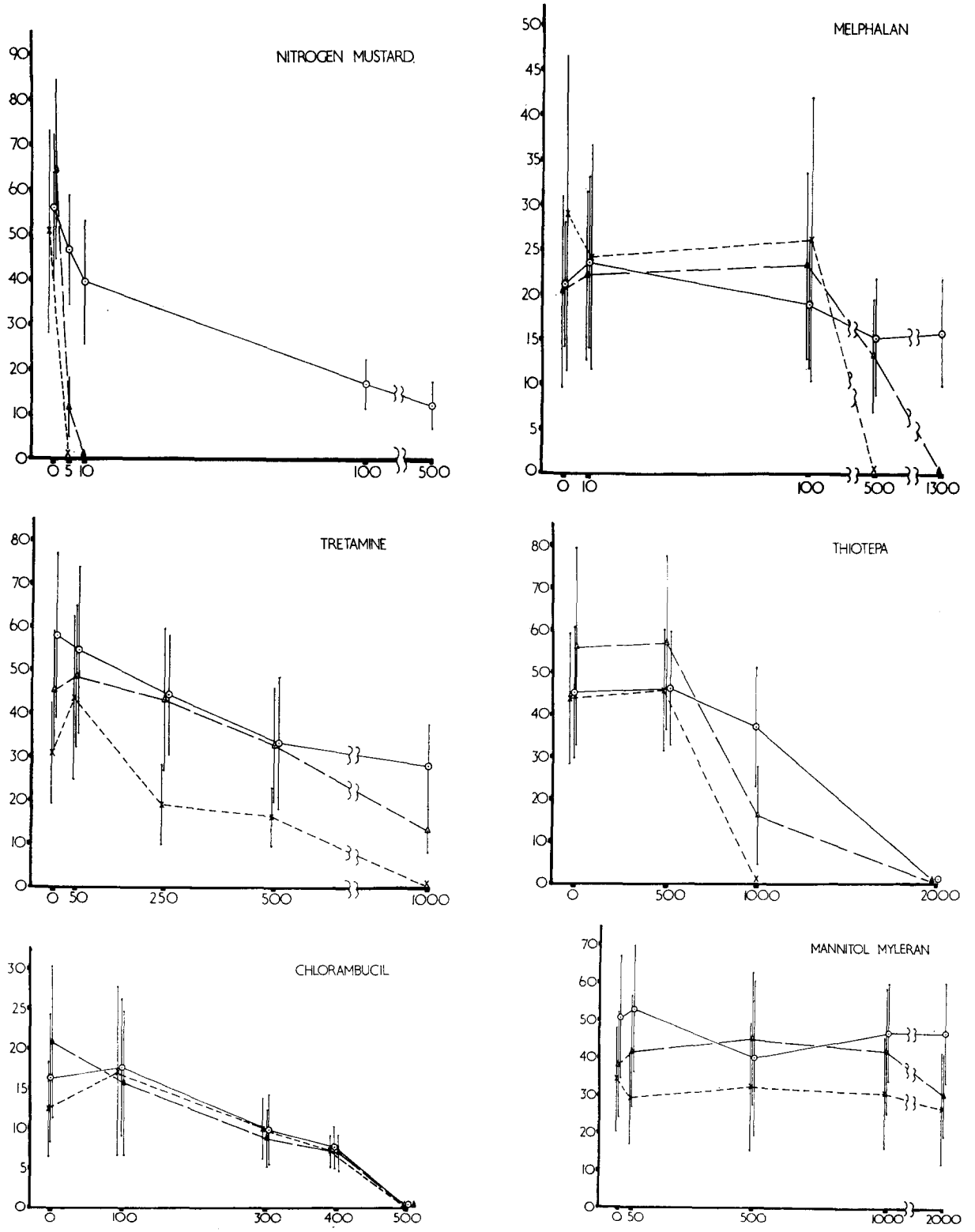
Chlorambucil and melphalan

In the chlorambucil and melphalan experiments, low grain counts per labelled cell and low percentages of labelled cells were found for cells incubated with the lower drug concentrations, and also for the diluent controls. This low level of labelling may have been due to the presence of propylene glycol.

Pretreatment

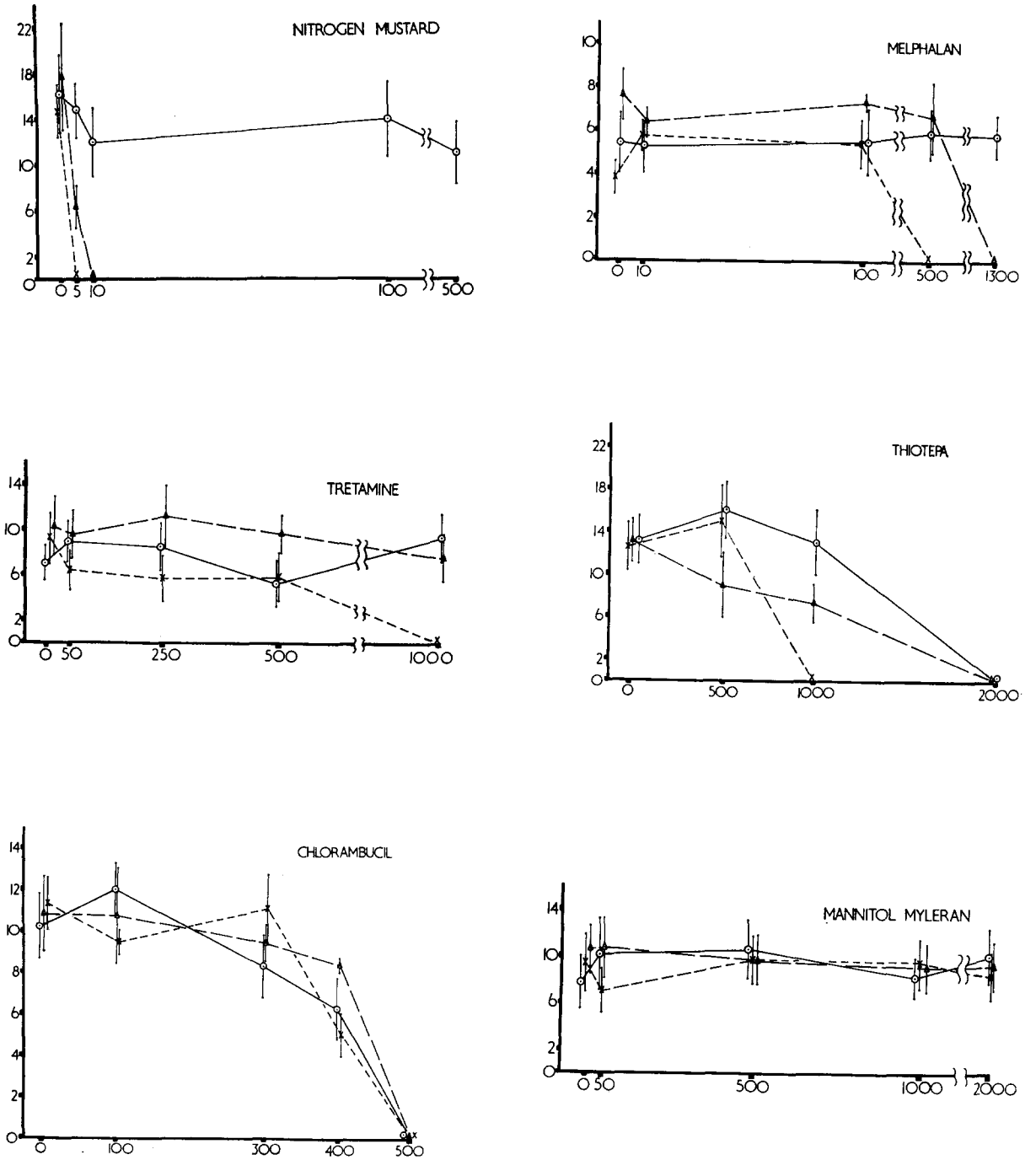
Treating the cells with drug before the addition of thymidine tended to decrease the uptake of thymidine by individual cells and reduce the proportion of cells labelled. In most cases, the longer the pretreatment time the lower the grain count at each drug concentration. The extent of this pretreatment effect depended on the drug. It was pronounced with nitrogen mustard and melphalan, moderate with thioTEPA and tretamine, and absent with chlorambucil. Five hundred microgrammes nitrogen mustard per 3×10^6 cells added immediately before the thymidine did not prevent thymidine uptake, whereas cells pretreated with as little as 10μg nitrogen mustard per 3×10^6 cells for 45 min showed no detectable uptake. After 1½ hr pretreatment, 5μg was sufficient to produce the same result. In contrast, cells incubated with chlorambucil incorporated the same quantity of thymidine whether the cells were incubated with drug and thymidine together or with thymidine added 45 min or 1½ hr after the drug.

Figs. 1–6. *Effect of selected alkylating agents on uptake of thymidine–H^3 by mouse thymocytes.*



(A. Effect on grains per labelled cell.)
Grains/labelled cell: Ordinate
μg drug/3 × 10^6 cells: Abscissa
⊙———⊙ no pretreatment
△—— ——△ ¾-hr pretreatment
×— — — —× 1½-hr pretreatment
•———• standard deviation

Figs. 7–12. Effect of selected alkylating agents on uptake of thymidine–H^3 by mouse thymocytes.



(B. Effect on percentage cells labelled.)

% cells labelled: Ordinate

μg drug/3 × 10^6 cells: Abscissa

⊙———⊙ no pretreatment

△—— ——△ ¾-hr pretreatment

×— — — —× 1½-hr pretreatment

•———• standard deviation

Table 1. *Drug concentrations (mM) causing "complete inhibition" and* 50% *reduction of thymidine uptake by mouse thymocytes*

Drug and pretreatment time (hr)	Complete inhibition		50% reduction	
	Based on results from grains/ labelled cell	Based on results from % cells labelled	In grains/ labelled cell	In % cells labelled
Nitrogen mustard				
0	>2·16	>2·16	0·13	>2·16
¾	>0·026 <0·043	>0·026 <0·043	0·015	0·013
1½	0·022	0·022	0·0087	0·0087
Tretamine				
0	>4·08	>4·08	3·77	>4·08
¾	>4·49 <5·51	>4·08	3·12	>4·08
1½	>2·65 <4·08	>2·45 <4·08	2·14	2·77
Mannitol myleran				
0	>4·93	>4·93	>4·93	>4·93
¾	>4·93	>4·93	>4·93	>4·93
1½	>4·93	>4·93	>4·93	>4·93
ThioTEPA				
0	>5·28 <8·81	>4·84 <8·81	5·88	5·64
¾	>4·62 <5·28	>5·06 <8·81	4·12	4·87
1½	>2·64 <4·40	>2·64 <4·40	3·57	3·41
Chlorambucil				
0	>1·23 <1·37	>1·15 <1·37	1·12	1·15
¾	>1·23 <1·37	>1·19 <1·37	0·68	1·19
1½	>1·23 <1·37	>1·15 <1·37	1·13	1·07
Melphalan				
0	>3·41	>3·41	>3·41	>3·41
¾	>1·70 <3·41	>1·55 <3·41	1·51	2·11
1½	>0·66 <1·31	>0·41 <1·31	0·83	0·73

Relative drug effectiveness

The probable molar concentration of each drug causing complete inhibition of detectable thymidine uptake, and the molar concentration of each drug causing a 50% reduction in the mean grain count per labelled cell and in the proportion of labelled cells from the control values, were judged from Figs. 1–12 (Table 1). Complete inhibition was said to have occurred when there were no more than four grains/cell. From these figures the drugs were placed in the order of their ability to interfere with thymidine uptake (Table 2). It was found that the order of effectiveness depended on which pretreatment time was selected. For example, with no pretreatment, chlorambucil was the most effective drug in preventing detectable uptake of thymidine. With 45 min pretreatment, nitrogen mustard was the most effective, with chlorambucil taking second place, and with 1½ hr pretreatment melphalan replaced chlorambucil as the second most active drug.

In most cases, the drug order obtained from reductions in uptake per labelled cell was the same as that obtained from reductions in the percentages of cells labelled. The one exception to this occurred when there was no pretreatment and 50% reduction from the control values was used as the end-point. In this case, chlorambucil was the most effective agent in reducing the proportion of labelled cells, but nitrogen mustard was the most effective in reducing the grain count per labelled cell.

DISCUSSION

It has been shown above that cell damage by these alkylating agents can be measured *in vitro*. Excepting mannitol myleran, all the alkylating agents examined decreased incorporation of exogenous thymidine into DNA of mouse thymocytes. Several drug actions could have caused this inhibition. Decreased thymidine uptake would occur: —

(*a*) if the thymidine to DNA pathway were directly blocked, or

(*b*) if damage took place before entry into the S phase, the thymidine to DNA pathway itself being undamaged, or

(*c*) if some of the cells were dead when the thymidine was added.

Each of these possibilities will be discussed in turn, with special reference to the present results and to the existing theories on the mode of action of alkylating agents.

a. *Direct inhibition of nucleic acid synthesis*

Inhibition of DNA synthesis by nitrogen mustard has been reported in many cell systems [for examples, see 11–18]. Some authors state that nitrogen mustard acts specifically on DNA synthesis, while others suggest that RNA synthesis is also affected and that protein synthesis may be reduced. These conclusions were reached with a variety of cell systems, both *in vivo* and *in vitro*, and with widely varying drug concentrations. There is some indication that the extent of the inhibitory action of nitrogen mustard depends on the drug concentration used. When Earle's 'L' cells were treated *in vitro* with 0·1μg / ml, DNA synthesis stopped 24 hr after drug treatment when the premitotic DNA level had been reached. RNA synthesis and protein synthesis were unaffected at this concentration but when the drug concentration was increased to 0·2–0·3μg / ml RNA synthesis and protein synthesis were also depressed and the cells eventually lysed [15]. Similarly, treating rabbits *in vivo* with 1·5–5 mg / kg nitrogen mustard and treating Earle's 'L' cells *in vitro* with 0·25μg / ml resulted in DNA synthesis being depressed more than RNA synthesis, whereas treating the more resistant Ehrlich ascites tumour cells *in vitro* with 50μg / ml resulted in both RNA synthesis and DNA synthesis being reduced to about 30–40% of the control values [19]. Some of the differences in drug action on metabolic pathways recorded by various authors can probably be explained in terms of differences in drug concentration used to treat tissues with different sensitivities to nitrogen mustard.

It has been shown that nitrogen mustard does inhibit nucleic acid synthesis in some cell systems, including the mouse thymocytes used in the present experiment. The mode of action of inhibition of nucleic acid synthesis by this drug is, however, disputed and a pretreatment effect would be compatible either with the idea that nitrogen mustard acts specifically on certain site(s) or with the idea that any reactive site may be attacked non-specifically [20, 21].

*Table 2. Order of drug effectiveness**

End-point	Pretreatment time (hr)	Based on results for per cent cells labelled	Based on results for grains per labelled cell
Complete inhibition of detectable thymidine uptake	0	C>others	C>others
	¾	N>C>Mp>$\overset{\text{Tr}}{\text{Th}}$	N>C>Mp>$\overset{\text{Tr}}{\text{Th}}$
	1½	N>Mp≥C>$\overset{\text{Tr}}{\text{Th}}$	N>Mp≥C>$\overset{\text{Tr}}{\text{Th}}$
50% reduction from control value	0	C>others	N>C>others
	¾	N>C>Mp>$\overset{\text{Tr}}{\text{Th}}$	N>C>Mp>Tr>Th
	1½	N>Mp>C>Tr>Th	N>Mp>C>Tr>Th

*Judged in terms of the molar concentration needed to produce the desired effect.

C — chlorambucil.

N — nitrogen mustard.

Mp — melphalan.

Tr — tretamine

Th — thioTEPA.

b. *Indirect inhibition of DNA synthesis resulting from a block in the cell cycle*

Any block in the cell cycle which prevented cells entering the synthetic phase (S phase) would eventually cause inhibition of thymidine uptake. Growth inhibition is not always associated with an immediate block in DNA synthesis. Thus, Ultmann *et al.* [22] obtained inhibition of mitosis in regenerating rat liver with doses of nitrogen mustard (1·9 mg/kg) which allowed the accumulation of both RNA and DNA beyond the control values. These increased values were thought to be due to the entry of fewer cells into the first mitosis after hepatectomy and, therefore, to an accumulation of cells with the premitotic DNA level. Similarly, growth inhibition of mouse tumours *in vivo* was produced with doses of nitrogen mustard (0·8 mg/kg) which had no inhibitory action on the incorporation of P^{32} into DNA [23]. Continued DNA synthesis up to premitotic levels in the presence of nitrogen mustard was also shown by Levis *et al.* [24] and by Brewer *et al.* [15]. These results indicate that nitrogen mustard can act in such a manner that DNA synthesis occurs normally up to premitotic levels but that further DNA synthesis is prevented, perhaps because the cells fail to divide.

Widner *et al.* [25], basing their arguments on the decrease in the mitotic index of transplanted rat tumours at various times after the rats were treated with nitrogen mustard, suggested several blocks in the cell cycle. It is generally accepted that nitrogen mustard acts during interphase though there are a few papers besides Widner's which suggest inhibition during metaphase (see Gelfant [26] for discussion and references). A block in the premitotic resting phase (G_2) is suggested by Layde and Baserga [27] and by Gelfant [26]. It is thought that cells that are in the early part of the G_2 phase when they are exposed to nitrogen mustard do not undergo mitosis. Cells in the latter part of the G_2 phase at that time are presumed to divide and pass through the following G_1 and S phases before being affected by the nitrogen mustard. This would explain the increase in dry mass [28] and the doubling of DNA content reported in cells treated with nitrogen mustard. RNA synthesis and protein synthesis are thought to be relatively unaffected.

If a G_2 block were taking place in the present experiment, a pretreatment effect would be expected. The longer the cells were in contact with the nitrogen mustard the greater the number of mitoses prevented and the fewer the cells entering the S phase. A lower percentage of tritium labelled cells would then result. To cause complete inhibition of thymidine uptake, the drug would need to be in contact with the cells for a pretreatment period equal to, or exceeding, the duration of the mitotic phase plus G_1 plus S plus any G_2 remaining after the block. Metcalf D. (personal communication) finds the cell cycles of large and medium thymocytes to be 6·8 and 8·2 hr respectively, with the S phases alone lasting 5·5 hr. The cell cycle of small thymocytes is much longer. Since a pretreatment period of 45 min with nitrogen mustard prevents detectable uptake of thymidine, the main action of nitrogen mustard on these cells cannot be a block in the premitotic resting phase. Moreover, appropriate concentrations of thioTEPA and chlorambucil prevented detectable thymidine uptake without pretreatment. The cells must have been damaged in the few minutes between the additions of drug and labelled precursor. The main action of these drugs under these experimental conditions cannot have been a block in the premitotic resting phase. The effect of nitrogen mustard has already been shown to depend on the concentrations used, and it is possible that with lower drug concentrations and longer pretreatment times such a G_2 block would be detectable in the present cell system.

A block in some part of the cell cycle other than the G_2 phase could result in a pretreatment effect but, again, complete inhibition of thymidine uptake would not be expected until a pretreatment time approaching the length of the cell cycle was used.

c. *Cell damage unrelated to DNA synthesis*

The cells could have been damaged at some site or sites not directly connected with thymidine uptake. Inhibition of thymidine uptake could have reflected cell death due to some unrelated damage. Using a partially synchronized cell suspension of Earle's 'L' cells, Walker and Hellcincr [29] studied the effect of nitrogen mustard on the viability of the different stages of the cell cycle. The synthetic phase was found to be the most susceptible. Assuming that this finding is not an artefact resulting from the chemical synchronization of the cells and that it can be applied to other cell systems, then it is possible that the mouse thymocytes in the S phase at the time of drug treatment were being preferentially killed. Reduced grain counts and lower percentages

of labelled cells resulting from increasing the pretreatment time with nitrogen mustard before the addition of thymidine would be compatible with this idea. The alkylating agents with the most pronounced pretreatment effects would presumably be those with the greatest damaging ability.

In conclusion, from observations of the pretreatment times needed to prevent detectable thymidine uptake, it appears that the main mode of action of these alkylating agents cannot have been a block in the cell cycle. Direct interference with the thymidine to DNA pathway (possibly by attack on the various enzymes involved) or non-specific interference with various metabolic processes with resulting cell death are suggested as possible main actions of these drugs on the mouse thymocytes in the present experiment.

Pretreatment effect

Some of the alkylating agents examined have been shown to have a more pronounced pretreatment effect (nitrogen mustard and melphalan) than others (tretamine and thioTEPA). No pretreatment effect was found with chlorambucil. Two possible causes of the pretreatment effect are postulated:

(1) It could be due to a time lag between the damage and the demonstrable effect on DNA synthesis

(2) It could reflect continuous and cumulative damage as long as the cell is in contact with the drug. These two possibilities are being examined.

Although the compounds studied are all alkylating agents, they are distinct chemical entities and as such cannot be assumed to react in a uniform manner. It is possible that the pretreatment effect simply reflects the time needed for sufficient drug to react and that this time varies from one drug to another. Published data on reaction rates are mostly derived from the study of isolated cell constituents in non-biological media. Under such conditions, competition by other nucleophilic substances and interference by cellular membranes, which would occur in intact cells, are absent. Further, the extent of drug adsorption to proteins in the present experiment is unknown. Israels and Linford [30] have shown that individual proteins have their own characteristic binding abilities and reaction rates with chlorambucil. Moreover, the chlorambucil results are difficult to explain in terms of relative rates of reaction since the drug appears to be as effective when added a few minutes before the thymidine as after 1½ hr pretreatment. Perhaps the chlorambucil is not acting as an alkylating agent in this system but prevents entry of thymidine by altering the cell surface. It may be significant that the compounds with the extreme effects, nitrogen mustard and chlorambucil, carry opposite charges when in solution, the ethyleneimmonium ion resulting from cyclization of nitrogen mustard acting as a cation and chlorambucil acting as an anion. Melphalan carries both a cationic and an anionic group and the compounds with an intermediate pretreatment effect are neutral in solution.

The possibility that the alkylating agents combine directly with the tritiated thymidine has also been considered [31, 32] but alkylation of thymidine is unlikely as sites of greater reactivity are available [33] and unpublished results [C. A. Brown] also appear to contradict this idea.

Mannitol myleran

The fact that mannitol myleran failed to show any inhibitory action on thymidine uptake, even at a concentration of 2 mg/ml, requires comment. Kellner and Németh [34] report that 1 mg mannitol myleran/ml had a marked *in vitro* effect after 24 hr on fibroblasts of chick embryo hearts, fresh Crocker sarcoma and HeLa cells. Two mg/ml caused cell destruction. Easty and Wylie [35] found that as low a concentration as 2·5–250 μg/ml had a cytotoxic effect on glioma cells. On the other hand, 33·8 mg/ml was found by Onesti and Woodliff [5] to have no effect on oxygen-uptake by chronic granulocytic leukaemia cells *in vitro*, and Reid [36] showed that 0·5 mg/ml had no effect on the *in vitro* synthesis of uridine nucleotides and glycogen by rat liver. This would indicate that different tissues have very different sensitivities to mannitol myleran and that mouse thymocytes may be among those that are relatively resistant.

Practical applications

The object of this study is to investigate an *in vitro* method which, it is hoped, will yield results reflecting the effects of drugs on growth of individual tumours *in vivo*. In the practical application of such a test, it is irrelevant whether the drugs prevent DNA synthesis directly or indirectly.

It has been shown that the order of effectiveness of different drugs appears to depend on the length of pretreatment and, in some circumstances, on the criterion used to measure the drug effect. Which, if any, of these timing procedures and criteria would yield results reflecting the effects of the drugs on the same cell system *in vivo* cannot be decided *a priori*. This can be determined only by performing parallel *in vitro* and *in vivo* studies. A further complication in the application of this test is that the majority of human tumour cells have a cell cycle longer than that of mouse thymocytes. Longer incorporation times would be needed to yield a countable number of cells labelled with thymidine.

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RÉSUMÉ

Dans le but de tester une méthode de mesure de la sensibilité individuelle des tumeurs aux agents chimiothérapiques in vitro, *l'auteur a étudié l'action de divers agents alkylants sur l'incorporation* in vitro *de la thymidine tritiée dans les thymocytes de souris. Des concentrations appropriées d'ypérite azotée, de tétramine, de thioTEPA, de chlorambucil et de melphalan diminuaient le nombre moyen de grains par cellule marquée et le pourcentage de cellules marquées, tandis que le mannitol-myleran (4,9 mM) n'avait aucun effet sur l'incorporation de la thymidine. En augmentant la durée d'incubation avec le produit, avant l'addition de thymidine, on intensifiait l'action du médicament, mais l'importance de cette intensification variait d'une substance à l'autre. Cet accroissement d'effet, en fonction de la durée d'incubation, était importante pour l'ypérite azotée et le melphalan, modérée pour la tétramine et le thioTEPA, et absente pour le chlorambucil. L'efficacité apparente des substances (déterminée par leur faculté de diminuer l'incorporation de thymidine par rapport aux contrôles) dépendait donc de la durée de l'incubation des cellules avec les agents chimiothérapiques.*

A partir de ces observations, l'auteur discute la validité des méthodes de mesure de la sensibilité des tumeurs in vitro, *comme moyen d'évaluer la sensibilité de ces mêmes tumeurs* in vivo. *Des études parallèles* in vitro *et* in vivo *sont nécessaires pour déterminer quelle méthode est capable d'apporter des renseignements valables pour la clinique. On pourrait aussi déterminer, de cette façon, s'il est préférable d'utiliser le pourcentage de cellules marquées ou le nombre de grains par cellule, comme critère d'évaluation.*

Des observations sur l'importance du temps d'incubation préalable avec la substance (aucune préincubation n'était nécessaire pour empêcher l'incorporation de thymidine avec le chlorambucil et le thioTEPA; 45 minutes de préincubation étaient nécessaires pour l'ypérite azotée et le melphalan; et une heure et demie pour la tétramine), on peut conclure qu'un arrêt, à un stade quelconque du cycle mitotique de la cellule, ne constitue pas le mode d'action principal de ces substances, dans les conditions expérimentales utilisées. Une interférence directe dans le processus métabolique conduisant de la thymidine à l'ADN, ou une interférence non spécifique dans divers processus conduisant à la mort de la cellule, sont proposées comme une explication possible du mécanisme d'action de ces substances.

SUMMARY

With a view to examining a proposed method for measuring in vitro *the sensitivity of individual tumours to chemotherapeutic agents, the effects of selected alkylating agents on uptake of tritiated thymidine by mouse thymocytes* in vitro *were investigated. Appropriate concentrations of nitrogen mustard, tretamine, thioTEPA, chlorambucil and melphalan decreased the mean number of grains per labelled cell and reduced the percentage of labelled cells, while mannitol myleran (4·9 m M) had no effect on thymidine uptake. Increasing the incubation time with drug before the addition of thymidine tended to enhance the drug action, but the extent of the pretreatment effect varied from one drug to another. The pretreatment effect was pronounced with nitrogen mustard and melphalan,*

moderate with tretamine and thioTEPA, and absent with chlorambucil. As a result of the pretreatment effect, the apparent effectiveness of the drugs (determined by their ability to reduce thymidine uptake relative to control values) depended on the pretreatment time used.

The application of these findings to an in vitro *method of determining sensitivity of individual tumours* in vivo *is discussed. Parallel* in vivo *and* in vitro *studies are needed to indicate which, if any, of these incubation procedures and methods of reading the results would be of value in a clinical assay and, to some extent, on whether percentage cells incorporating thymidine or uptake per labelled cell should be used as the criterion of drug effect.*

From observations on the pretreatment time needed to prevent detectable uptake of thymidine (no pretreatment necessary with chlorambucil and thioTEPA, 45 min needed with nitrogen mustard and melphalan and 1½ hr with tretamine), it is concluded that a block at any one point of the cell cycle cannot be the main mode of action of these drugs under these experimental conditions. Direct interference with the thymidine to DNA pathway or non-specific interference with various metabolic processes resulting in cell death are suggested as possible modes of action of these drugs.

ZUSAMMENFASSUNG

Um eine vorgeschlagene Methode, welche dazu bestimmt ist die Empfindlichkeit einzelner Tumoren verschiedenen chemotherapeutischen Mitteln gegenüber in vitro zu untersuchen, zu überprüfen, wurde der Einfluss dieser Mittel aufddie Einverleibung von mit Tritium markiertem Thymidin in Mäusethymozyten in vitro studiert. Entsprechende Mengen von N-Lost, Tretamin, ThioTEPA, Chlorambucil und Melphalan führten zu einer Verminderung der Zahl der Körner pro markierte Zelle und des Prozentsatzes der markierten Zellen, während Mannitol-Myleran (4,9 mM) keinen Einfluss auf die Thymidineinverleibung hatte. Die Verlängerung der Inkubationszeit mit den Chemotherapeutica, ehe Thymidin hinzugegeben wurde, erhöhte die Wirksamkeit der letzteren, aber diese Wirksamkeit war uneinheitlich. Die Wirksamkeit dieser Vorbehandlung war sehr ausgesprochen im Falle von N-Lost und Melphalan, mässig mit Tretamin und ThioTEPA und abwesend mit Chlorambucil. Das Resultat der Vorbehandlung lässt den Schluss zu, dass die scheinbare Wirksamkeit, gemessen an ihrer Fähigkeit die Thymidineinverleibung im Vergleich zu den Kontrollwerten zu vermindern, von der Dauer der Vorbehandlung abhängt.

Die Anwendung dieser in vitro-Methode der Bestimmung der Sensibilität individueller Tumoren in vivo wird besprochen. Parallele Untersuchungen in vivo und in vitro sind notwendig um festzustellen, ob diese Inkubationsmethoden und die Anwendung ihrer Resultate klinisch verwendbar sind und zwar insbesondere um festzustellen, ob der Prozentsatz der Zellen, welche Thymidin einverleiben, oder ob die Einverleibung pro markierte Zelle als Prüfstein für die Wirksamkeit eines Medikamentes verwendet werden soll.

Aus der Bestimmung der notwendigen Vorbehandlungszeit, um eine erkennbar Thymidineinverleibung zu verhindern (mit Chlorambucil und ThioTEPA ist keine Vorbehandlung notwendig, mit N-Lost und Melphalan 45 Minuten und mit Tretamin 1½ Studnen) wird der Schluss gezogen, dass unter den genannten Versuchsbedingungen eine einfache Blockierung an irgendeinem Punkte des Zellteilungszyklusses nicht die Hauptwirkung dieser Medikamente ist. Die direkte Beeinträchtigung des Thymidin-DNA-Weges oder unspezifische Eingriffe in den Zellstoffwechsel, welche zu dem Zelltode führen, werden als mögliche Wirkungsmechanismen dieser Mittel in Erwägung gezogen.

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Aldolase Isozymes in Cancer*

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INTRODUCTION

THE subject of this report is the study of multiple molecular forms of aldolase, the enzyme which catalyses the aldol cleavage of fructose-di-phosphate, in human and experimental cancer, in order to verify the hypothesis of a modification of the repartition of these isozymes in some cancers.

The works of Hers [1], Peanasky and Lardy [2], Blostein and Rutter [3, 4] and ourselves [5] have shown that muscle and liver aldolases are two distinct entities, with some similar properties. The main difference between the two enzymes lies in their relative specific activities toward two substrates: the elective substrate, fructose-1-6-diphosphate (F-d-P) and a second substrate fructose-1-phosphate (F-1-P).

Rutter has called aldolase "A" the aldolase which acts primarily on F-d-P and only slightly on F-1-P (Ratio F-d-P/F-1-P$>$50); and aldolase "B", the enzyme which catalyses the aldol cleavage of the two substrates at about equal rates (Ratio F-d-P/F-1-P$=$1).

In muscle, aldolase "A" is present almost to the exclusion of aldolase "B"; in liver, the reverse is true. In mammalian tissues, the distribution of the two types of aldolase varies widely with the organ, as we have shown in studying the ratio of two activities: F-d-P/F-1-P [5].

METHODS

The measurements of aldolase activities were carried out using a modification of the Sibley and Lehninger technique [6]; the results are expressed in international units. The experimental hepatoma used was the one isolated by Zajdela [7]; it is an ascites hepatoma transplanted to adult Wistar rats. The samples of human hepatoma were removed a few hours after death; the samples of normal human liver were removed by biopsy or after death: we have verified that the ratio F-1-6-P/F-1-P was not altered *post mortem*. Experimental cancer of spleen was obtained from Tran Ba Loc in Swiss mice, by transplantation of Sarcoma ST4. Originally, this reticulum cell sarcoma was induced by repeated injections of chloramphenicol into adult mice [8].

RESULTS

Table 1 shows the results that we have obtained with Jean-Claude Dreyfus and Georges Schapira. In human hepatomas, the aldolase activity ratio is very different from that in normal adult liver:

In eight cases of human hepatomas (in which the diagnosis was confirmed by histological examination), we found a ratio between 2·2 and 13, with a mean value of 5·5, therefore much higher than normal [9, 10].

We have also studied some other pathological human livers; the ratio remained normal in almost all cases. Only in cirrhosis was this ratio slightly higher. But calculation shows that fibrosis, although frequent in cancer, is quite insufficient to explain the anomaly of hepatoma.

We confirm also the results of Hers who described a similar biochemical abnormality of aldolase in liver of children with fructose intolerance [11]. The table shows a mean ratio of 5·5 in eight cases of this congenital disease; this ratio is very close to that found in cancer. In foetal liver the aldolase activity ratio was not 1 but 2 to 6 [9, 11, 12].

In order to understand the significance of these results, we looked for similar modifications of aldolase in other tissues. We have studied, the spleen with Tran Ba Loc, in which the

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Table 1. *Ratio of aldolases activities in liver: F-1-6-P/F-1-P*

	Human	Rat
Normal adult	1·0	1·0
Foetal	2–3 (4–7 months)	2–6 (16th–20th day)
Various pathological conditions	1·0	1·0
Cirrhosis	1·6	
Benign fructosuria	1·0	
Fructose intolerance	5·5	
Hepatomas	5·48±1·17	37

normal ratio of aldolase activities is about 9.

We found an abnormality of aldolase apparently contrary to that which we described in cancerous liver: in the spleen of mice the normal ratio was 8·97. In cancerous spleen, this ratio was much lower: 4·5 on the average. Moreover, we found in spleen of foetal mice ratios of aldolase activities lower than in the normal adult (3·5 and 6·4 in two experiments) [13].

Table 2. *Normal and cancerous spleens (mice)*

	Aldolases		
	F-1-6-P	F-1-P	Ratio
Normal adult	1·76 ±0·055	0·195±0·015	8·97±0·5
Cancerous	0·759±0·27	0·162±0·009	4·51±1·54
Foetal (mean of two experiments)	2·24	0·47	4·7

DISCUSSION

The contrast between the results in liver and in spleen cancer may be explained as follows: Normal liver is of type B (with F-d-P activity much higher than F-1-P). Consequently, in both, the specific type of aldolase is diminished. In hepatoma, the pure type B is replaced by a mixture of "B" and "A" characterized by an intermediary ratio; in spleen where there is principally aldolase "A", it is this aldolase "A" which partially disappears in cancer. One should also notice that the abnormality found in cancerous liver and in cancerous spleen is similar to the foetal state.

It seems therefore that cancer tends to reproduce the foetal state; and that there is perhaps in foetal or embryonic state a mixture of the two types of aldolase, "A" and "B" in all the organs. During differentiation, the biosynthesis of either "A" or "B" would be predominant: for instance the type "B" is necessary in liver for fructose metabolism.

We may propose the hypothesis of the repression of the most differentiated and preponderant molecular form of aldolase in cancer.

SUMMARY

There are two different types of aldolase in mammalian tissues, the main difference between these isozymes is the relative activity towards the second substrate of aldolase, fructose-1-phosphate (F-1-P). In normal liver the ratio of the activities towards fructose di-phosphate (F-d-P) and towards fructose-1-phosphate, is near 1, *because normal liver contains almost exclusively the type of aldolase characterized by a ratio F-d-P/F-1-P=*1.

In human primary liver cancer (and also in experimental hepatoma), this ratio is raised to a mean value of 5.5.

On the contrary, muscle contains almost exclusively the type of aldolase with a ratio

greater than 50. *The two types of aldolase exist in normal spleen, but principally the latter type.*

In spleen of normal mice, we have found an aldolase ratio of 8.97*; in experimental cancer (reticulum cell sarcoma of spleen) we have found the ratio lowered to* 4·51.

These results are discussed in presenting the hypothesis of the repression, in cancer, of the most differentiated enzyme.

RÉSUMÉ

Les tissus des mammifères possèdent deux types d'aldolase; la principale différence entre ces isozymes est l'activité relative vis-à-vis d'un second substrat de l'aldolase, le Fructose-1-phosphate. Dans le foie normal, le rapport des activités vis-à-vis du Fructose-di-phosphate (F-d-P) et du Fructose-1-phosphate (F-1-P), est proche de 1, *car le foie normal contient presque uniquement le type d'aldolase caractérisé par un rapport F-d-P/F-1-P=1. Dans le foie humain porteur de cancer primitif (et aussi dans l'hépatome expérimental) ce rapport s'élève à une valeur moyenne de* 5.5.

Le muscle, au contraire, contient presque uniquement le type d'aldolase caractérisé par un rapport F-d-P/F-1-P supérieur à 50.

Ces deux types d'aldolase existent dans la rate normale, mais surtout le dernier type. Chez la souris normale, nous avons trouvé dans la rate un rapport moyen d'activité aldolasique de 8,97. *Dans le cancer expérimental (réticulosarcome de la rate) nous avons trouvé que ce rapport était abaissé en moyenne à* 4,51.

Ces résultats sont discutés d'après l'hypothèse de la répression, au cours du cancer, de l'enzyme la plus différentiée.

ZUSAMMENFASSUNG

In Säugetiergeweben befinden sich 2 *verschiedene Typen der Aldolase: der Hauptunterschied zwischen beiden Isoenzymen besteht in ihrer relativen Aktivität dem zweiten Aldolasesubstrat gegenüber, nämlich dem Fruktose-1-Phosphat (F-1-P). In der normalen Leber ist das Verhältnis zwischen den Aktivitäten gegenüber dem Fruktose-di-Phosphat (F-d-P) und dem Fruktose-1-Phosphat beinahe gleich* 1, *da normales Lebergewebe fast nur den Typ der Aldolase, welcher durch das Verhältnis F-d-P/F-1-P=1 charakterisiert wird, enthält.*

In Fällen von primärem Leberkrebs (und auch bei dem experimentellen Hepatom) erhöht sich dieses Verhältnis durchschnittlich auf 5,5.

Im Gegensatz hierzu enthält Muskelgewebe fast ausschliesslich den Typ der Aldolase, bei dem dieses Verhältnis höher als 50 *ist.*

Beide Aldolasetypen befinden sich ebenfalls in normalem Milzgewebe, aber in der Hauptsache handelt es sich um den 2. *Typ.*

In normalem Mäusemilzgewebe findet man ein Aldolaseverhältnis von 8,97*; in experimentellem Krebsgewebe (Reticulo-Sarkomder Milz) ist das Verhältnis auf* 4,51 *herabgesetzt.*

Die Autoren besprechen ihre Resultate und unterstellen als Hypothese, dass in Krebsgewebe das differenziertere der zwei Enzyme unterdrückt wird.

Das Verhältniss der beiden Aldolaseaktivitäten der Leber steigt beim primitiven Krebs. Es sinkt in der milz mit Retikulosarkom. Die Hypothese wonach das beim Krebs meist differenzierte Enzyme unterdrückt ist könnte diesen Unterschied erklären.

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Methotrexate and Folic Reductase in Human Cancer*†

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THE PRECISE biochemical functions of methotrexate in man are not fully known. It is thought to block the enzyme dihydrofolic reductase or folic reductase which, as shown in Fig. 1, reduces folic acid with the aid of NADPH to 7, 8, dihydrofolate at a slow rate, and thence to 5, 6, 7, 8 tetrahydrofolate at a fast rate [1, 2]. The reduced form is then acted upon by formate-activating enzyme to give compounds of importance (Fig. 2) in the biosynthesis of purines and pyrimidines, as well as in serine-glycine interconvensions [3, 4].

Methotrexate binds almost irreversibly with folic reductase so that the amount of drug taken up can be used as a measure of enzyme present [5]. The assays used in our studies involved the measurement of (1) formylated compound [6] (2) the diazotizable amine formed from dihydro and tetrahydrofolate [7] and (3) the spectrophotometric changes between DHFA and THFA, plus the oxidation of NADPH [8].

The distribution of folic reductase in animal tissues does not parallel their mitotic activity [9]. Hence, there must be other uses for the enzyme or storage in the liver and kidney [10]. Table 1 shows the uneven distribution of enzyme among tissues of various species, with the monkey and man showing little enzyme in any tissue examined. The data in Table 2 shows the distribution of enzyme in several human tissues; as in the monkey, it is very much lower than is seen in smaller animals [11]. A tumor tissue in which a considerable amount of folic reductase was found was Wilms' tumor of the kidney, as shown in Table 3. The amounts found here, although greater than in other human tissues, were only one tenth of that in rat kidney. In spite of the large amounts of target enzyme for the drug, thirty cases of Wilms' tumor treated with methotrexate to evidences of biological activity, failed to show antitumor effect. Hence, the mere presence of target enzyme in tumor cells is not enough to insure an antitumor effect.

Leukemic white cells also have appreciable amounts of folic reductase [12]. The acute leukemia cells, particularly, have enzyme and this has been thought to be the reason for their sensitivity to methotrexate. Resistance to methotrexate has been induced in several animal leukemias and greatly elevated levels of enzyme noted [13]. However, in man, we were unable to relate initial levels of enzyme with drug sensitivity [14]. As can be seen in Table 4, the presence or absence of enzyme did not predict for drug responsiveness and, although the level of enzyme rose following treatment with methotrexate, this did correlate with the development of drug resistance.

We then attempted to look at drug uptake as a factor involved in methotrexate sensitivity and resistance, using human peripheral white cells and the method outlined in Table 5. We were able to add different substances at step two to study their effect upon transport and by observing the loss of label after resuspension in fresh medium; efflux could also be studied. As shown in Fig. 3, uptake is a temperature-

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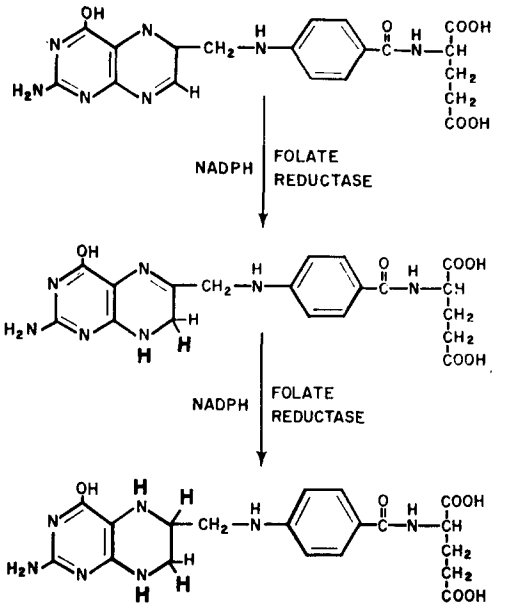

2. TETRAHYDROFOLATE + ATP + FORMATE ⇌ N^{10} FORMYLTETRAHYDROFOLATE + ADP + Pi

FORMYLTETRAHYDROFOLATE SYNTHETASE

Fig. 1. *Assays for folic reductase.*

1. Dihydrofolate + Triphosphopyridine Nucleotide (red).
   ↓ FOLIC REDUCTASE
   Tetrahydrofolate + Triphosphopyridine Nucleotide

2. Serine + Tetrahydrofolate → N^5, N^{10}-Methylene THFA + Glycine
   (SERINE HYDROXYMETHYLASE)

3. Deoxyuridine-5′-Monophosphate + N^5, N^{10}-Methylene THFA
   ↓ THYMIDYLATE SYNTHETASE
   Thymidine-5′-Monophosphate + Dihydrofolate

Fig. 2.

Table 1. *Distribution of folic reductase by tissue and species**

Specie	Liver	Kidney	Thymus	Intestinal Mucosa	Spleen	Lung	Heart	Brain
Rat	1·17	0·37	0·76	0·58	0·46	0·15	0	0
Rat (6 days old)	1·27	0·48	0·64	—	0·98	0·25	Trace	0
Mouse	0·85	0·82	0·39	0	0·21	0	Trace	0
Rabbit	0·87	0·15	0·93	0·12	1·33	0·12	0	0
Guinea Pig	1·20	0·70	0·09	0·29	0·26	Trace	0	0
Hamster	0·99	0·98	—	0·32	0·15	0	0	0
Dog	0	0·25	—	Trace	—	Trace	0	0
Cat	0·17	Trace	Trace	0	0·08	0	0	0
Sheep	0·66	Trace	—	Trace	Trace	—	0	0
Sheep (fetal)	0·61	0·08	0·35	—	0·29	—	Trace	Trace
Calf (1 day)	0·43	0·37	0·25	0·16	0·11	Trace	0	0
Monkey	Trace	0	0	0	0	0	0	0
Chicken	0·75	—	—	0·30	—	Trace	0	0

*mμ moles equivalent methotrexate per gram.

Table 2. Folic reductase activity of human tissue

Organ	Specimen	Total	λ=560 mμ*		λ=355 mμ†		λ=340 mμ‡	
		No.	No.	+	No.	+	No.	+
Brain	Normal	2	2					
	Astrocytoma	7	5		2	1		
	Meningioma	2	2					
	Pituitary	1	1					
Tonsils and Adenoids	Normal	3	3		3			
Thymus	Normal	3	3					
Larynx	Carcinoma	3	3					
Lung	Normal	3	3					
	Carcinoma	5	3				2	1
Liver	Normal	13	7	1	3	2	3	
	Diseased	3	3	2			2	1
	Carcinoma	2	1	1	1	1		
Spleen	Normal	4	4				2	
	Leukemia	6	4		2	1	2	
	Dyscrasias	4	4				1	
Esophagus	Carcinoma	2	1		1	1		
Stomach	Normal	6	4				2	
	Carcinoma	5	5				1	
Small Intestine	Normal	4	4					
Colon	Normal	13	12		9		3	1
	Carcinoma	14	9	3	2	1	2	
Breast	Normal	4	4		3		1	1
	Carcinoma	8	8		1			
Kidney	Normal	4	4	1	2		1	
	Wilms'	12	12	10			6	5
	Carcinoma	3	3	1	1		1	
Testicle	Normal	6	3		3		2	1
	Seminoma	2	2				1	1
	Carcinoma	1	1	1			1	1
Prostate	Normal	1	1				1	
	Hypertrophy	3	3					
	Carcinoma	2	1		1		2	2
Ovary	Normal	7	7	2				
	Carcinoma	5	5	1				
	Cyst	1	1					
Uterus	Normal	5	3		2		1	
	Sarcoma	5	5	3				
	Carcinoma	4	2		2			
Cervix	Normal	5	5					
	Adenomyosis	1	1		1			
	Carcinoma	4	4	4				
Placenta	Normal	9	7	2	6			
	Mole	2	1	1	1			
Pancreas	Normal	1	1					
	Carcinoma	1	1					
Skin	Melanoma	2	2					
Gall Bladder	Cholecystitis	1			1			
Muscle	Normal	8	8		3			
Buttock	Liposarcoma	1	1					
Nerve	Ganglioneuroma	2	2					
	Neuroblastoma	4	4	1				
Adrenal	Carcinoma	1	1					
Lymph Node	Granuloma	1	1					
	Lymphangioma	1	1					
	Lymphadenitis	1	1					
Retina	Tumor	1	1					
Totals		223	194	32	50	7	35	13

*Assayed for the appearance of a diazotizable amine [7].

†Assayed by the conversion of tetrahydrofolate product to N^5, N^{10} methenyl tetrahydrofolate [6].

‡Assayed by decrease in absorbancy [8].

Table 3. Folic reductase activity of specimens of kidney tissue including Wilms' tumor

No.	Specimen No.	Pathology	Folic reductase*
Wilms'			
1	CMC 360	Wilms' tumor	0·63
2	CMC 340	Wilms' tumor	0·23
3	CMC 239	Wilms' tumor	0·062
4	CMC 201	Wilms' tumor	0·67
5	CMC 219a	Wilms' tumor	Active[1]
6	CMC 147	Wilms' tumor	Trace
7	CMC 232	Wilms' tumor	0
8	CMC 244	Wilms' tumor	0
9	CMC 355	Metastatic Wilms' tumor to liver	0·67
10	CMC 249a	Metastatic Wilms' to liver	0·015
11	CMC 134c	Metastatic Wilms' to lung	Active
12	CMC 214	Metastatic Wilms' from pelvis	Active
Normal			
13	CMC 157	Normal kidney	0
14	CMC 219b	Uninvolved kidney[1]	Trace
15	PBBH 24b	Uninvolved kidney	0
Pathological			
16	B145	Hydronephrotic	0
17	B81	Renal cell carcinoma	0
18	B102	Transitional cell carcinoma	0
19	USPH1	Renal cell carcinoma	Trace

([1]) Note uninvolved kidney specimen (CMC 219b) has one-eighth the level of the tumor.
*mμ moles equivalent Methotrexate per gram protein.

Table 4. Conversion of folic acid to tetrahydrofolic acid by homogenates of white blood cells from patients with acute leukemia in relation to methotrexate therapy

	Folic reductase activity			
	Absent		Present	
Methotrexate	−	−	+	+
Remission	−	+	−	+
Myelogenous	7/13	0/0	5/5	1/1
Lymphocytic	15/17	0/1	8/9	7/7

Table 5. Method of measurement of MTX transport by leukocytes;

1. Isolation of cells: dextran sedimentation and osmotic lysis of rbc.
2. Incubation in 10 volumes of 150 *mM* buffer at pH 7·2+MTX-H^3.
3. Dilution with cold buffer; centrifugation.
4. Resuspension and centrifugation of cells.
5. Extraction and determination of intracellular MTX-H^3.

Table 6. Effect of methotrexate analogs on transport of MTX-H³

Analog at 10 μg/ml	MTX-H³ Uptake
No additions	100%
Non-radioactive MTX	35%
Dichloro-MTX	30%
Aminopterin	45%
Citrovorum Factor	33%
Folic Acid	70%
N^{10}-Methyl Folate	80%
Triamterene (100 μg/ml)	300%

Normal wbc incubated for 15 min at 37° in tris buffer at pH 7·2 containing 0·1 μg/ml of MTX-H³.

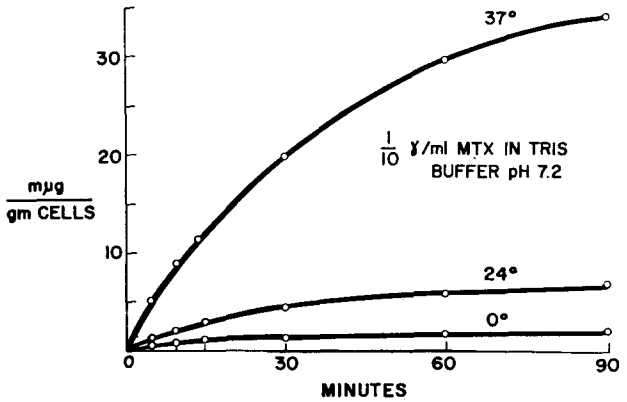

Fig. 3. Uptake of methotrexate-H³ by normal leukocytes.

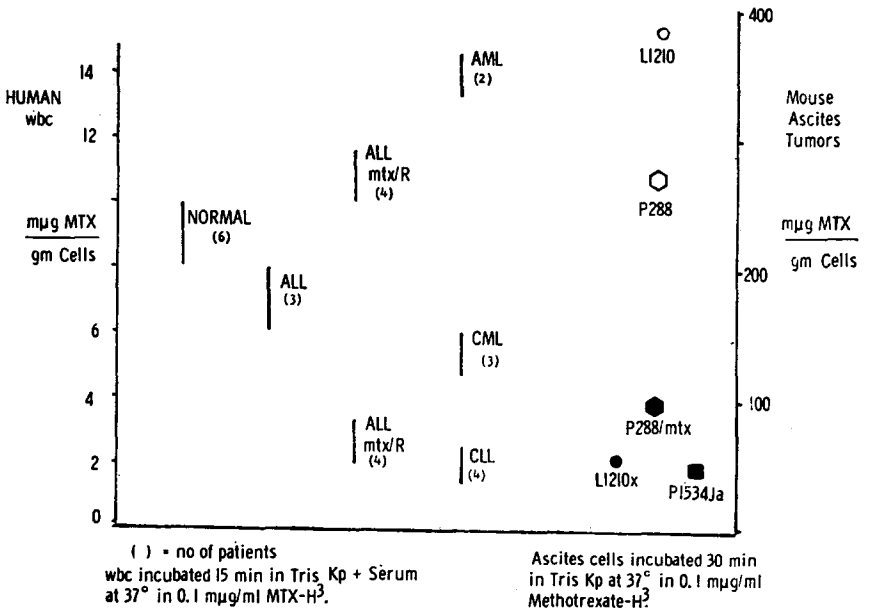

Fig. 4. Uptake of methotrexate by leukemic cells.

ALL, acute lymphatic leukemia; *CLL, chronic lymphatic leukemia;*
AML, acute myelogenous leukemia; *CML, chronic myelogenous leukemia.*

dependent process, no net concentration against a gradient is obtained, and the amount taken up increased with the outside drug concentration. This type of transport has been called "facilitated diffusion" by Danielli [15].

Table 6 shows that the process of uptake is inhibited by several compounds. Citrovorum factor is notable in this respect and some explanation of its protective action against methotrexate may be found here; L-folate, but not D-folate, is also inhibitory. Of considerable interest is the observation that the pteridine diuretic, "Triamterene," enhances uptake of methotrexate [16]. We found this effect to be more pronounced at zero degrees than at thirty-seven degrees, suggesting some damage to cell wall which was repairable metabolically. Examination of Triamterene-treated cells showed considerable morphologic changes. We are continuing to investigate this phenomenon vigorously, since drugs which would facilitate methotrexate uptake might render insensitive cells susceptible to drug action.

Figure 4 indicates the relation between drug uptake and methotrexate sensitivity in a series of mouse and human leukemias. We had previously shown a lack of correlation between enzyme and drug sensitivity in man and also that, although the level of folic reductase was high in the cells of mouse leukemia with drug-induced or "acquired" resistance, there was no correlation between folic reductase level and native or "primary" drug responsiveness [17].

As you can see, the levels of drug uptake are low in resistant mouse leukemia, even though the folic reductase level is low, as in the naturally methotrexate-resistant line P1534, or high in the drug-induced forms of L1210x and P288/MTX. Hence, among both types of resistance, that is, the primary or natural and the induced or acquired, low drug uptake seems to be a common factor.

Our findings are also seen in Fig. 4 for the small number of human cells so far examined. The methotrexate-resistant chronic leukemias show low uptakes. The acute lymphatic leukemias show uptakes which fall into two classes as resistance develops — some with low uptake, some with high. The ones with high uptake, we would postulate, should have high enzyme levels. Our preliminary results indicate that 0·1 mg/ml of methotrexate in the incubating medium is able to inhibit all DHFR in normal cells in 15 min at 37°. This is a level obtainable in man by the injection of a massive single dose of methotrexate. If the cells have low uptake or high enzyme levels, we find the drug cannot completely inhibit the enzyme present.

RÉSUMÉ

1. *La réductase de l'acide folique existe dans les tissus des animaux, à des niveaux et des concentrations qui ne reflètent pas l'acivité mitotique de ces tissus.*

2. *Chez le Singe et chez l'Homme, le niveau d'activité de l'enzyme est beaucoup plus bas que chez les Rongeurs et n'est pas parallèle à la sensibilité au méthotrexate, comme le montre l'étude de la tumeur de Wilms.*

3. *La captation du méthotrexate marqué au tritium, par les globules blancs humains, se fait selon un processus de facilitation de la diffusion.*

4. *Les niveaux d'activité de l'enzyme ne permettent pas, à eux seuls, de prédire la sensibilité ou la résistance au médicament, dans les leucémies des Rongeurs et de l'Homme. On peut prédire la résistance de l'étude combinée du transport de la drogue et des niveaux d'activite de l'enzyme.*

5. *Des méthodes qui augmentent la captation du méthotrexate, y compris des schémas posologiques qui amènent de fortes concentrations dans le milieu extracellulaire et l'utilisation de substances qui facilitent le transport, peuvent augmenter l'activite antimorale du méthotrexate.*

SUMMARY

1. *We have found folic reductase to exist in animal tissues at levels and concentrations which do not reflect tissue mitotic rates.*

2. *In monkey and man the level of enzyme is much lower than in rodents and does not parallel drug sensitivity as in the Wilms' tumor, for example.*

3. *Uptake of tritiated methotrexate by human white blood cells is by a process of facilitated diffusion.*

4. *Enzyme levels alone cannot predict drug sensitivity or resistance in rodent or human leukemia, but resistance can be predicted as a result of both drug transport and enzyme levels.*

5. *Methods which increase methotrexate uptake, including dose schedules which create high external concentration, and the use of transport-facilitating drugs, may increase the antitumor effectiveness of methotrexate.*

ZUSAMMENFASSUNG

1. *Die Folsäure-reduktase ist in tierischen Geweben in Mengen und Konzentrationen nachzuweisen, die in keinem Zusammenhang mit der Zellteilungszahl stehen.*

2. *Bei dem Affen und dem Menschen ist die Enzymmenge viel niedriger als bei Nagetieren und sei geht nicht parallel mit der Drogenempfindlichkeit wie in Fällen von Wilm'schen Tumoren.*

3. *Die Aufnahme von tritriummarkiertem Methotrexat durch menschliche weisse Blutkörperchen geschieht durch erleichterte Diffusion.*

4. *Die Enzymkonzentration allein erlaubt es nicht die Drogenempfindlichkeit oder Resistenz vorauszusagen und zwar weder in Fällen von menschlicher noch von Nagetierleukämie, aber die Resistenz kann vorausgesagt werden unter Berücksichtigung des Drogentransportes und der Enzymkonzentration.*

5. *Methoden, die die Methotrexeataufnahme erhöhen, wie Dosierungen welche eine hohe externe Konzentration erzielen, kombiniert mit transporterliehcternden Drogen, könnten unter Umständen die antitumorale Wirkung des Methotrexats erhöhen.*

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Use of an Indirect Antiglobulin Consumption Test to Detect Isoantibodies Following Human Cancer Cell Homotransplants*

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INTRODUCTION

THIS PAPER reports the modification and use of an indirect antiglobulin consumption (IAGC) test for the detection of isoantibodies following the homotransplantation of human cancer cell lines in healthy recipients and in patients with cancer. Reproducibility, sensitivity, difficulties and merits of the technique will be considered. The antibody data will be compared with those obtained previously by use of the tanned erythrocyte agglutination technique [1–3]. These studies are confirmatory of the results obtained by the tanned erythrocyte technique and indicate that the cellular antigens responsible for the IAGC reaction are present at the cell membrane.

METHODS

Serologic methods

The concept of measuring decreases in antibody titer to determine amount of antigen was introduced by Weiner and co-workers [4] to study globulin levels in cerebrospinal fluid. This was a direct antiglobulin consumption test. The extension of this concept to the detection and measurement of cell antigens was developed by several investigators [5–8]. After the antigenic material, e.g. leukocytes, had been allowed to react with the serum under investigation the unadsorbed antiserum was removed and the amount of antibody (globulin) that had reacted was determined indirectly by determining how much antiglobulin would react with the antigen-antibody complex. This is an indirect antiglobulin consumption test. The technique used in the present investigation is based on these same principles but differs considerably from previously published methods.

In general outline the technique consists of the following steps: preparation of suspensions of live tissue-cultured cells, reaction of the test serum with these cells; repeated washings to remove unadsorbed serum; reaction of these cells with a rabbit antihuman globulin serum; titration of residual antiglobulin. The loss of antiglobulin titer reflects the amount of globulin adsorbed onto the tissue-cultured cells from the serum under study. A difference between the amount of globulin adsorbed from a serum obtained before homotransplantation and a serum collected from the same person after homotransplantation is considered to be due to isoantibody.

The present test is modified from previous IAGC tests in two ways. First, the primary antigen-antibody reaction is carried out in a single mixture of reagents, rather than in a series of mixtures in which antigen or antiglobulin is serially diluted. This was necessitated by the prohibitively large quantity of test serum or fresh tissue-cultured cells that would be needed for multiple dilutions. Second, measurement of the amount of antiglobulin before and after "consumption" was accomplished by use of a tanned erythrocyte technique

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(agglutination of sheep erythrocytes which have been treated with tannic acid and then coated with human globulin) based on the method of BOYDEN [9]. This second modification was principally a matter of convenience, because the tanned erythrocyte technique was being used concurrently in this laboratory for other studies, whereas other methods of titrating the antiglobulin (such as Rh positive cells and a human anti-Rh serum) would have required additional reagents.

It seems quite possible that in this technique the HEp 2 cells are agglutinated by the reaction of the antiglobulin with the isoantibody that coats them but if such agglutination occurred it was obscured by the spontaneous cohesiveness of the washed tissue-cultured cells and hence could not be utilized as an indicator of isoantibody adsorption.

Reagents used for the serologic test. Technical details are as follows.

Isotonic saline: a solution of 0·15M sodium chloride.

pH 7·2 phosphate buffered saline (PBS): a solution made by mixing nine parts of isotonic saline and one part of 0·15M pH 7·2 phosphate buffer (approximately 7·6 ml of 0·15M Na_2HPO_4 plus 2·4 ml of 0·15M NaH_2PO_4, but exact amounts were adjusted as necessary to give pH 7·20).

pH 6·4 phosphate buffered saline: a solution made by mixing nine parts of isotonic saline and one part of 0·15M pH 6·4 phosphate buffer (approximately 2·8 ml of 0·15M Na_2HPO_4 plus 7·2 ml of 0·15 ml NaH_2PO_4). This buffer was prepared fresh daily.

0·5% normal rabbit serum saline (NRS saline): 0·15M sodium chloride solution with 5 ml of normal rabbit serum per liter. The normal rabbit serum had been inactivated by heating at 56°C for 10 min and absorbed with sheep erythrocytes.

Except where otherwise specified, *the above four solutions also contained* 10 *mg tetrasodium ethylene diamine tetra-acetate* (*EDTA*) *per liter.* The EDTA was used because of previous observations that these procedures reduced non-specific agglutination in the tanned erythrocyte agglutination technique, presumably by removal of divalent cations, but its importance for the present technique was not investigated.

EDTA solution (for harvesting tissue cultures): a solution of 200 mg EDTA per liter in pH 7·2 phosphate buffered saline.

Antiglobulin: a rabbit antihuman globulin serum prepared by Knickerbocker Biologics, a division of Pfizer Laboratories, New York City, for use in the Coombs test.

Human gamma globulin: Cohn's fraction II of pooled human serum distributed by Parke-Davis and Company under the trade name "Immu-G". This contained approximately 165 mg of gamma globulin per ml. This preparation was diluted 1 : 8 with 0·15M NaCl to form a stock solution.

Tannic acid solution: Analytical grade tannic acid from Mallinckrodt Company was made up as a 0·1% stock solution in deionized water and stored at – 20°C in aliquots sufficient for a week's work.

Preparation of tissue-cultured cells for use as test antigens. All cell lines used as test antigens were grown in stationary "milk dilution" bottles (11 × 3 cm surface area per side) as monolayers on the glass with no other substrate. The medium was 80% Eagle's minimal essential medium [10] plus 20% newborn "a-gamma" calf serum (Hyland Laboratories, Los Angeles) with added penicillin (200 U/ml) and streptomycin (200 gamma/ml). After discarding culture medium, the monolayers were rinsed with 15 ml of EDTA solution to wash away residual culture medium. Then another 10 ml of the same EDTA solution was added to each bottle and they were incubated for 15–20 min at 37°C to free cells from the glass surface. (Trypsin was equally satisfactory for harvesting the cultures but was used in only one study, as mentioned in Results). The cells were then harvested by shaking gently and pipetting up and down 10 times to separate cell aggregates into single cells. The cell suspension was then spun down and washed twice with NRS-saline. Centrifugation was at approximately 80 × g for 4 min. The washed cells were resuspended in 10–15 ml of 0·5% NRS-saline and left at room temperature for 5 min so that aggregated cells would sediment out. The resulting single-cell suspension was again spun down at 80 × g for 4 min to determine packed cell volume, and then resuspended in nine parts of 0·5% NRS-saline to make a 10% cell suspension. This was the "test antigen".

Reaction of test sera with test antigen. Equal volumes (0·2 ml) of the test antigen (usually HEp 2 cells) and an undiluted test serum were pipetted into the bottom of Kahn tubes (75 mm × 10 mm dia.) using care to avoid depositing the reagents on the walls of the test tube. The tubes were covered with parafilm to retard evaporation and incubated at room temperature for 1 hr with intermittent shaking. This allowed reaction of isoantibody (plus any non-specifically reacting serum components)

with the tissue-cultured cells — the primary reaction of this serologic method. Then 1·0 ml NRS EDTA saline was added to the mixtures. The tubes were again shaken, then centrifuged at 80 × g for 3–4 min at room temperature, and the fluid discarded. The pellet of cells was resuspended in NRS EDTA saline again, and the suspension centrifuged. This washing procedure was repeated 6 times to eliminate unadsorbed serum components. After the final washing the cells were centrifuged for 6–7 min at 80 × g to give a compact button and the supernatant fluid was eliminated as completely as possible by micropipetting.

Consumption of antiglobulin by the sensitized test cells. The tissue culture cells, after reacting with the test sera in the above procedure, were mixed with 0·4 ml of antiglobulin appropriately diluted with NRS EDTA saline, and incubated at room temperature for 40 min with repeated shaking. The dilution of the antiglobulin was 1 : 6 except in some of the exploratory studies. The mixtures were then centrifuged at approximately 500 × g for 5 min and the supernatant antiglobulin (now "consumed antiglobulin") was harvested with micropipettes and the cells (now presumably coated successively with isoantibody and antiglobulin) were discarded. The supernatant was usually titrated immediately for residual antiglobulin (see below) but could be stored in the refrigerator without change in titer for at least 20 hr.

Preparation of globulin-coated erythrocytes. Sheep erythrocytes were washed in EDTA saline until the supernatant was clear and then made up to 2·5 per cent suspension in the same solution. This suspension was then mixed with an equal volume of 1 : 20 dilution of the stock solution of tannic acid (i.e., final tannic acid concentration was 1 : 40,000). After incubation for 10 min in the 36°C water bath the "tanned erythrocytes" were washed once with EDTA saline and resuspended in unbuffered 0·15M NaCl to make a 2·5 per cent suspension. Then one volume of this tanned erythrocyte suspension was mixed with two volumes of the human globulin solution, diluted so as to contain approximately 300 μg of globulin (200 μg of gamma globulin) per ml and the mixture was incubated for 30 min at room temperature. These globulin-coated erythrocytes were then washed 3 times with NRS EDTA saline to remove unadsorbed serum components and finally resuspended to 2·5 per cent in the same diluent.

Titration of residual antiglobulin. Serial two-fold dilutions of each test preparation of "consumed antibody" (see second preceding paragraph) were prepared with NRS EDTA saline as the diluent. Since the antiglobulin had already been diluted 1 : 6 in previous steps, 0·3 ml was added to 0·7 ml of the diluent to give a starting concentration of 1 : 20. From this, 7 or 8 two-fold dilutions were made using a 0·5 ml carry-over in Kahn tubes. 0·05 ml of the globulin-coated erythrocyte suspension was then added to each tube. The racks of tubes were shaken thoroughly and incubated overnight at 4°C. Sedimentation patterns were routinely read after overnight incubation and graded stringently according to Stavitsky's criteria [11]. Only clearcut agglutination patterns with no circumferential ring formation were graded as 2+. Definite agglutination but with a thin ring was graded 1+, but a wide ring was graded as ± and was not considered as a positive reaction. An additional reading was usually made after resuspending the erythrocytes following the overnight incubation, and allowing sedimentation to occur a second time. It was also possible to make readings after sedimentation at room temperature for 1–1½ hr prior to incubation in the refrigerator. These readings were usually identical and never differed by more than one tube from the routine readings. The greatest dilution showing a 1+ or greater agglutination pattern was taken as the residual titer of the antiglobulin. The following controls were included in every test: a routine titration of the antiglobulin, and complete IAGC tests with a known positive human serum, a known negative human serum, and a 25 per cent normal rabbit serum in place of human serum. The residual antiglobulin titer in this normal rabbit serum control was the baseline for all calculations of globulin consumption. It was always either identical with the routine titration or one tube lower than the routine titration.

Calculation of isoantibody titers. The difference in antiglobulin titers between a test serum and the normal rabbit serum was the measure of antiglobulin consumption and was expressed as "number of tubes difference". The difference of globulin consumption between the pre-homotransplant serum specimen and the post-transplant specimen of an individual recipient were taken as the isoantibody titers. (See Results).

Other methods

Homotransplant recipients were of two types. Healthy recipients were adult male volunteers studied at the Ohio State Penitentiary in collaboration with Drs. R. H. Brooks, C. A. Doan, and A. G. James of the Ohio State

Table 1. Non-specific adsorption of serum globulin by HEp 2 cells in IAGC test. These sera were collected before homotransplantation or were from persons who did not receive homotransplants

Source of sera	Age		Sex		Tubes difference from no-human serum control								
	Range	Mean	M	F	0	1	2	3	4	5	6	7	Mean
Healthy persons	21–63	34	73	2	2	14	35	17	4	2	1		2·2
Cancer Patients	34–62	46	1	7				2	5	1			3·9
Sick persons without cancer	43–83	61	12	4			1	1	3	3	6	2	5·0

University Medical School. The other recipients were women with advanced cancer (six with epidermoid cervix cancer and two with ovarian adenocarcinoma) studied at Memorial Cancer Center in collaboration with Drs. A. Brunschwig and A. G. Levin.

Homotransplants were subcutaneous injections of 1–5 million tissue-cultured cells suspended in 0·5 ml of isotonic salt solution (usually Gey's salt solution). Each recipient had from two to six simultaneous injections including from one to four human cancer cell lines. Many of the healthy recipients were also given repeat homotransplants after intervals of several months. None of the cancer patients in the present study group received a second set of transplants. The technique of transplantation and the course of growth and rejection of the homotransplants has been discussed in previous publications [12–14] and additional material will be published elsewhere.

Sera from several persons who did not receive homotransplants were also studied. Age, sex, and clinical status are recorded in Table 1.

Sera were collected from the healthy volunteers on the day of homotransplantation and usually at 2, 4 and 6 weeks thereafter. In one study a serum was collected at 3 weeks instead of at 2 and 4 weeks, and in some studies the last specimen was obtained at 7 or 8 weeks instead of 6. Sera from the cancer patients were obtained at less regular intervals, as dictated by their periods of hospitalization and out-patient appointments. Sera were stored at $-10°$ to $-20°$C and were inactivated (56°C for 30 min) before use in the serologic studies.

Four tissue culture cell lines were used in the serological studies as the "test antigens": HEp 2, a human epidermoid larynx carcinoma cell line from a patient of blood group O, Rh positive [15, 16]) J-111, a cell line cultivated from peripheral blood of a monocytic leukemia patient of blood group A, Rh negative [17]; RPMI-41, a cell line originating from ascitic effusion in a patient of blood group O, Rh negative, with osteogenic sarcoma [18]; and rat nephroma, a tumor cell line derived from a spontaneous embryonal nephroma of a rat (unpublished work of V. I. Babcock, this laboratory). HEp 2 was used as the test antigen for each serum. The other three cell lines were used only in the comparative studies described in Results. In addition to these three human cell lines (HEp 2, RPM-41, and J-111), the following cell lines were used for homotransplantation: MAC 21 from a patient of blood group A, Rh negative with colon adenocarcinoma [19]; Detroit 6, from bone marrow of a lung cancer patient of blood group A, Rh positive [20]; RPMI-212, from ascitic effusion due to mesothelioma in a patient of blood group A, Rh positive [18]; Ovary 2, from an ovarian adenocarcinoma of a patient of blood group A, Rh positive [21]; Amnion B, an established culture line derived from a homotransplant nodule which developed from subcutaneous transplantation of a primary culture of pooled human amnions [2]; and a fibroblast line grown from a human fetus and maintained in passage for 6–9 months when used for the transplants but subsequently lost. (The blood groups of the patients from whom these cultures originated were provided by the originators of the cell lines by personal communication).

Cell types used for transplants are indicated for each individual referred to in figures or tables, but this information is not presented for all recipients because in these and previous studies there was no evident relation between cell line transplanted and antibody response, or between ABO blood group of donor and recipient, and because previous studies too showed extensive cross-antigenicity between all of these cancer cell lines.

RESULTS

Non-specific Reactions. When sera from persons who had not received homotransplants were studied by the indirect antiglobulin consumption (IAGC) test using HEp 2 cells, some adsorption of globulin onto the tissue-cultured human cells almost always occurred. This "non-specific" adsorption was occasionally very striking—causing as much as six to seven tubes difference from the non-human-serum controls (Table 1). That is, when HEp 2 cells were mixed with the serum, they adsorbed enough globulin to consume a large part of the subsequently added antiglobulin.

This reaction is non-specific in the context of this study because it is not a result of the cancer cell homotransplants. It may be that it is also non-specific in the sense that it is not an antigen-antibody reaction. Investigation of this question was not an objective of the present studies but two pertinent experiments were carried out. In one type of experiment, sera for which the magnitude of non-specific and specific reactions were known from previous tests, were studied by the IAGC technique using both the tissue-cultured rat nephroma cells and the human HEp 2 cells in parallel tests. It was found that globulin was adsorbed from the pretransplantation sera onto the rat cells to essentially the same degree as with HEp 2 cells, indicating that the reaction was not limited to human cells. However, sera containing isoantibody showed adsorption onto rat cells only to an extent consistent with the non-specific reaction which was seen in pretransplantation serum specimens from the same individual. In the other type of experiment, aliquots of four sera known to contain isoantibody were adsorbed by either rat nephroma cells or HEp 2 cells and then were tested, in parallel with unadsorbed aliquots of each, by the IAGC test with HEp 2 cells. (Adsorption was for 1 hr at room temperature using one part packed washed tissue-cultured cells to two parts undiluted serum). Adsorption by HEp 2 cells eliminated all or almost all reaction in the subsequent IAGC test, apparently eliminating both isoantibody and the non-specific reaction. In contrast, adsorption by the rat cells diminished reaction in the subsequent IAGC test by zero to three tubes, eliminating the non-specific reaction but not the isoantibody reaction.

The magnitude of this non-specific reaction was lowest in sera collected from healthy persons, and was slightly less in the cancer patients than in the patients with chronic diseases other than cancer (Table 1). These three groups differed greatly in age and sex, so it cannot be concluded that the non-specific reaction is a result of disease.

Obviously, this phenomenon of "non-specific" adsorption must be taken into consideration when using the IAGC reaction for detection of isoantibody induced by homotransplants. Accordingly, the adsorption observed in the pre-transplantation serum was taken as the baseline for each individual person, and only differences from that baseline were considered to be the result of isoantibody formation. A very high non-specific adsorption precluded the titration of isoantibody, and because of this difficulty the antibody response in recipients with non-neoplastic diseases was not pursued. Furthermore, the validity of these isoantibody titers is dependent upon the constancy of this baseline non-specific reaction for each person during the 6–8 weeks period during which serum samples were obtained. In these studies there is no direct proof of such constancy, but constancy is suggested by the fact that in those recipients in whom isoantibody was not demonstrated, the degree of "non-specific" reaction was usually constant for the duration of the study. The amount of non-specific reaction, as judged by the pretransplant serum specimen is indicated for each individual in Table 3 and in the legends for figures.

Reliability of the technique. The *reproducibility* of the technique was checked repeatedly. Serial specimens from six healthy homotransplant recipients, totaling thirty-one sera, were tested by two persons working independently a week apart. Among the thirty-one serum specimens, twenty gave identical results, eight gave one tube higher and three gave one tube lower isoantibody titers in the second study. On another occasion seven sera were tested by two persons independently but simultaneously, using the same reagents and the same HEp 2 cell suspension. Comparing the results of one worker against the other, reactions were identical for two sera, showed one tube greater consumption by two sera, and one tube less by three sera. Still another group of thirty-two serial serum specimens from seven healthy homotransplant recipients were each tested on three, four or five occasions, over a period of 5 months. All replicate tests gave identical antibody titers for twenty sera, a maximum spread of one tube for eight sera, and a spread of two tubes for four sera. Another three sera — one from a healthy man who had not received homotransplants and two from

healthy men who had received repeated transplants were each tested thirteen times — being used as known negative and known positive controls. All except three of the thirty-nine endpoints fell within one tube deviation from the modal value for the same serum, and the three exceptions deviated only two tubes from the mode. Thus it can be concluded that the IAGC technique has a high degree of reproducibility for differences in excess of one tube, but that no significance can be attached to "one-tube titers".

The possibility was considered that the calf serum used in the culture medium might remain on the surface of the tissue culture cells used for the test antigen even after repeated washings [22], and either be a site of reaction with the test serum, or interfere with the isoantibody reaction by blocking antigenic sites on the cell surface. However, neither calf serum nor rabbit serum interfered with the IAGC test, as evidenced by essentially identical results when six serial serum specimens from both of two healthy homotransplant recipients were mixed 2 : 1 with undiluted normal rabbit serum or agamma calf serum, incubated at room temperature for 1 hr, and then titrated against HEp 2 cells in parallel with the test serum only. In addition, it was shown that serum from homotransplant recipients gave no reaction when calf serum was used as the test antigen in the tanned erythrocyte agglutination technique.

Although EDTA was used to harvest the tissue-cultured cells from the bottles in all the studies reported in this paper, twenty sera from four of the homograft recipients were tested against cells harvested with trypsin (0·25% in 0·15M NaCl) with essentially identical results.

The *reliability* of the IAGC test for the detection and quantitation of isoantibody responses to homografts is dependent upon ability to measure accurately the differences between the reactions of pretransplantation and post-transplantation serum specimens. It would be anticipated that the use of very small amounts of the antiglobulin serum in the penultimate step of the technique (the step where the antiglobulin is allowed to react with the isoantibody-coated cells) would increase the chance of detecting consumption of the anti-globulin, i.e., would increase sensitivity of the technique, but would also diminish the range over which changes could be quantitated. The use of a serial two-fold dilution series for titration of residual antiglobulin in the last step of the technique might be expected to measure progressively smaller differences between successive tubes. To investigate these relationships a series of studies was carried out using different dilutions of antiglobulin serum in a model reaction system in which various known amounts of human gamma globulin were incubated with the antiglobulin serum. (No tissue culture cells were used in this study. The entire amount of human globulin which was added to each tube was available for reaction with the antiserum. No visible precipitate formed and no attempt was made to remove the globulin-antiglobulin complex). The experimental data are summarized in Fig. 1. They confirm that range and sensitivity depend on the amount of antiserum used in the test. Moreover, they demonstrate that for each of the antiglobulin concentrations that

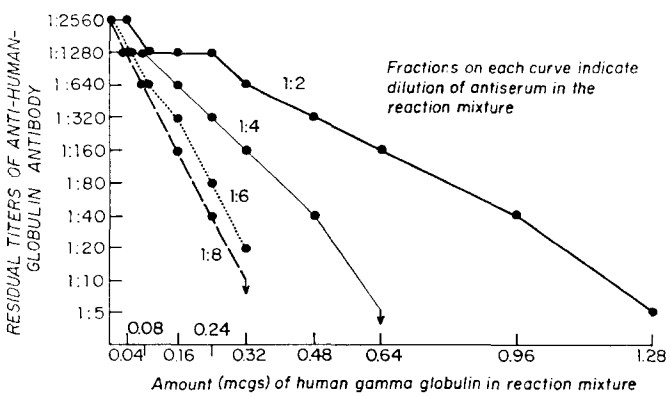

Fig. 1. Relationships between amount of globulin available for reaction, and the amount of antiglobulin consumed in the IAGC test. Successive two-fold differences in the titer of residual antibody in the reaction mixture (vertical axis) measured essentially equal amount of globulin in the reaction mixture (horizontal axis).

was studied, the relationship between the amount of globulin and the residual titer of the antiglobulin was nearly linear. For example, with 1 : 4 dilution of antiglobulin in the reaction mixture, each 0·08 mg increment of gamma globulin resulted in a further one tube drop in residual antiglobulin titer. This semilogarithmic relationship was unexpected and is unexplained, but was observed with each of the four globulin concentrations that were tested. If it can be assumed that the results obtained with this model system (in which the globulin was in solution) can be transposed to the IAGC test (in which the globulin was adsorbed to the tissue-cultured cells), it can be concluded that in the IAGC test an *equal difference* in number of tubes reflects essentially *equal amounts* of globulin adsorbed by the test cells regardless of where in the dilution series the difference occurs. This conclusion is strengthened by the essentially identical isoantibody titers obtained in repeated studies of the same individuals in spite of variations from 1 : 640 to 1 : 2560 in the titer of antiglobulin in the controls.

Isoantibody responses following homotransplants in healthy recipients. The IAGC test gave no convincing evidence of serum isoantibody against HEp 2 cells following primary transplantation of tissue-cultured cell lines. Among fifty-two healthy individuals whose sera were studied at 2, 4, and 6 weeks after primary transplant less than 10 per cent showed a two-tube titer of isoantibody and none showed more (Table 2). Even if one tube titers are considered, only 50 per cent of recipients had demonstrable isoantibody.

After a second homotransplant there was a significant increase in antibody response. About half of all recipients of a second series of transplants had an isoantibody titer of two tubes or more at 2 or 4 weeks after transplantation, 47 per cent had three tubes or more, and 33 per cent had four or five tubes. Nearly 80 per cent had one-tube differences, and although "one-tube titers" are not significant in individual tests, the difference in frequency of entirely negative tests in the primary and repeat transplant groups (26/52 versus 4/19 respectively) can hardly be attributed to chance ($p = 0{\cdot}03$).

After three sequential transplants isoantibody responses were very similar to the secondary response. These data are presented in more detail in Table 2. Mean antibody titers were near the maximum by 2 weeks, were maximal at 4 weeks and had dropped to below the 2 week level by 6–8 weeks. The homotransplants were injected in a series of nine studies over the period 1956–1963. A considerable variety of cell types was used (see Methods) including epidermoid carcinoma, adenocarcinoma, and sarcoma cell lines. The frequency and magnitude of responses was found to be quite consistent in all nine studies. Antibody responses in some individual healthy recipients are illustrated in Table 3 and Figs. 2 and 3.

Isoantibody responses following homotransplants in cancer patients. Of the eight cancer patients studied following primary homotransplants

Table 2. Frequency and titer of serum antibody responses following primary and repeat homografts of human cancer cell lines in healthy recipients. Indirect antiglobulin consumption technique with HEp 2 cells as test antigen. Fractions indicate number of sera with the indicated reaction over the total number of sera tested

Injection sequence	Weeks after injection	Isoantibody titers (Number of tubes difference from day 0 of same transplant)						Percent with detectable antibody		
		0	1	2	3	4	5	1 tube or more	2 tubes or more	3 tubes or more
1°	0	56/56								
	2	32/52	15/52	5/52				38	10	0
	4	26/52	23/52	3/52				50	6	0
	6–8	34/56	21/56	1/56				42	2	0
2°	0	22/22								
	2	8/21	3/21	2/21	1/21	5/21	2/21	62	48	38
	4	4/19	5/19	1/19	3/19	3/19	3/19	79	53	47
	6–8	5/18	6/18	2/18	3/18	0/18	2/18	72	39	28
3°	0	9/9								
	2	2/8	1/8	1/8	2/8	2/8		75	63	50
	4	1/8	1/8	2/8	1/8	3/8		100	75	50
	6–8	2/6	1/6	1/6	1/6	1/6		67	50	33

Table 3. Comparison of isoantibody titers using different cell lines as the test antigen

Recipient		Homotransplants		Serum	Test antigen, IAGC technique					
					HEp 2		J-111		RPMI 41	
Number	Age Sex	Sequence	Cell lines*	Date†	Non sp‡	Isoab‡	Non sp	Isoab	Non sp	Isoab
				Healthy recipients						
111–929	21M	1°	HEp 3	14	2	2	2	2	1	0
			MAC 21	28		2		2		1
			& Det 6	42		1		1		
111–643	29M	1°	HEp 3	14	2	2	1	2		
			MAC 21	28		2		2		
			Det 6	42		2		2		
107–163	26M	1°	HEp 2	14	2	1	2		1	
			MAC 21	28		1		1		1
			RPMI 212	42		1				
		2°	HEp 3	14	3	5	3	4	2	7
			MAC 21	28		5		4		7
			Det 6	42		5		4		5
105–759	50M	1°	Det 6	42	4	1	3	2		
		2°	HEp 3	14	4	4	4	3		
			MAC 21							
			RPMI 41	28		4		2		
			Det 6	42		3		2		
110–282	24M	1°	HEp 2	14	2	0	1		1	
			MAC 21	28		0		1		1
			Det 6	42		0				
		2°	HEp 3	14	3	5	2	3	2	5
			MAC 21	28		3		3		4
				42		3		3		
106–811	52M	1°	J-111							
			Ov 2	Not tested						
			Det 6							
		2°	HEp 3	14	4	4	2	4	2	7
			MAC 21	28		4		3		7
			RPMI 41	42		3		3		7
112–433	34M	1°	HEp 2	Not tested						
		2°	HEp 3	14	2	4	2	4		
			MAC 21	28		3		3		
			Det 6	42		2		2		
Cancer patient recipients										
25–28–25	62F	1°	RPMI 41	6	4	1	3	1		
			Amn B	11		2		1		
				18		3		3		
25–65–58	39F	1°	RPMI 41	24	4	1	3	0		
			Fibrobl.							
20–82–17	41F	1°		9	4	1	3	1		
				53		2		1		
25–69–80	47F	1°	HEp 2	25	4	3	3	2		
			Fibrobl.	40						
25–13–63	56F	1°	RPMI 41	24	3	2	2	2		
			Amn B	33		1		1		
25–80–89	40F	1°	HEp 2	9	5	0	3	2		
				18		0		0		
				58		0		0		
				70		0		0		
25–89–51	34F	1°	HEp 2	33	3	2	3	2		
22–07–51	46F	1°	RPMI 41	19	4	0	3	2		
			Fibrobl.	26		0		1		

*All the cell lines indicated for a given transplant study (primary or repeat) were injected at the same time.

†"Serum date" is indicated as number of days after transplantation.

‡Non-specific adsorption ("Non sp") indicates results of day 0 (pretransplantation) serum. Isoantibody "(Isoab") is recorded as the *difference* from the day 0 result of the same injection sequence with the same test antigen. When more than one study was done with one test antigen on a series of serum specimens, the most representative one is tabulated, but results from different studies are never mixed or averaged. Tests of different cell lines as the test antigen were done on different days.

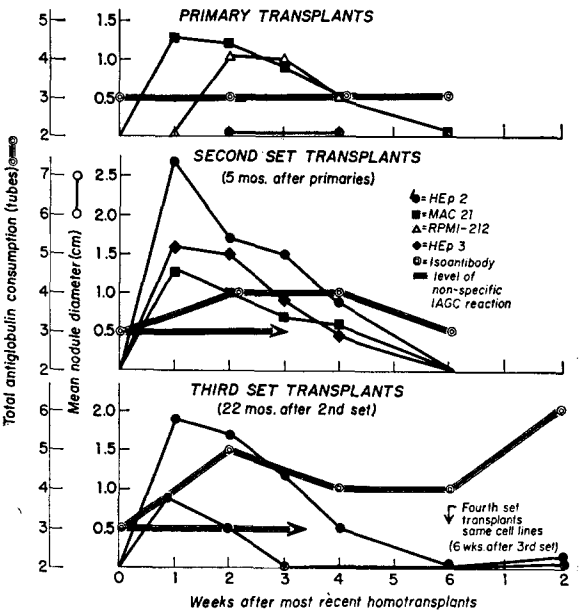

Fig. 2. Isoantibody responses and course of homograft growth and rejection following 1°, 2°, 3°, and 4° implants of various cell lines in a healthy recipient (No. 109–822). The primary transplants were completely rejected by 6 weeks but no antibody was detected. Subsequent transplants show accelerated rejection and successively higher levels of antibody.

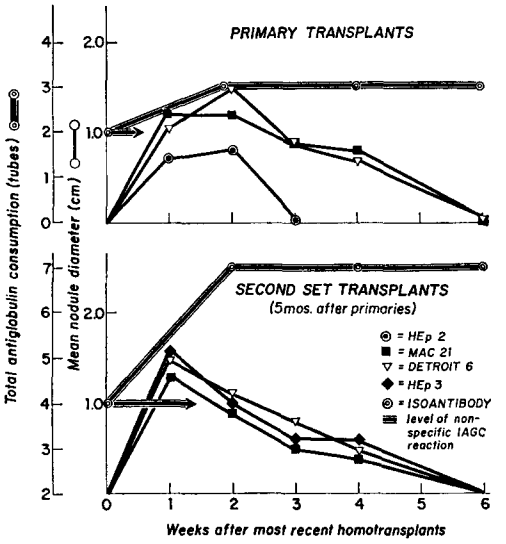

Fig. 3. Isoantibody responses and course of homograft growth and rejection following 1° and 2° implants of various cell lines in a healthy recipient (No. 107–163). Following the 1° transplants antiglobulin consumption increased by one tube. Following the 2° transplants (which demonstrate accelerated rejection) antibody titers was three tubes.

tissue-cultured cancer cells, five showed two tubes or more of antibody, and two showed three tubes, against HEp 2 cells in at least one of the serum specimens collected between 2 and 8 weeks after transplantation. This frequency of two- and three-tube antibody responses to primary homotransplants is notably greater than among normal recipients, but the group is so small that the difference cannot be considered meaningful, except as proof that these patients were able to produce serum antibodies in response to these allogeneic tissue antigens.

No data are available by IAGC technique on antibody to *repeat* homografts in recipients with cancer. Data from all the cancer patients are included in Table 3. Figures 4 and 5 illustrate the antibody response in two of the cancer patients in graphic form.

Cross-reactions. Sera from seven of the healthy recipients and eight of the cancer patients were also studied using J-111 cells as the "test antigen". Four of the healthy men were also studied using the RPMI 41 cell line. The results (Table 3) were generally the same as with the HEp 2 cell line. This cross-reaction with cancer cells of diverse tissue origins was also observed in previous studies using the tanned erythrocyte agglutination technique [1, 2]. Tissue culture cell lines of normal origin were not studied by the IAGC technique.

Relationship of serum antibody to homograft rejection. In healthy recipients the rise and fall of isoantibody, generally paralleled the growth

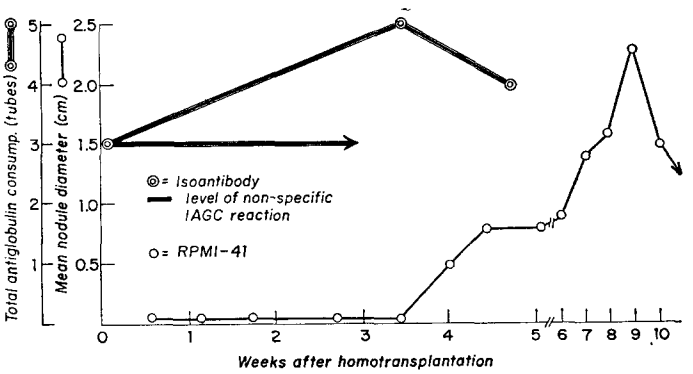

Fig. 4. Isoantibody response and course of growth and rejection of homotransplant nodule following subcutaneous transplantation of RMPI 41 cells in a recipient with advanced cancer of the cervix (No. 25-13-61). There is evidence of antibody during the third and fourth weeks but the transplant nodule continued to grow until the ninth week.

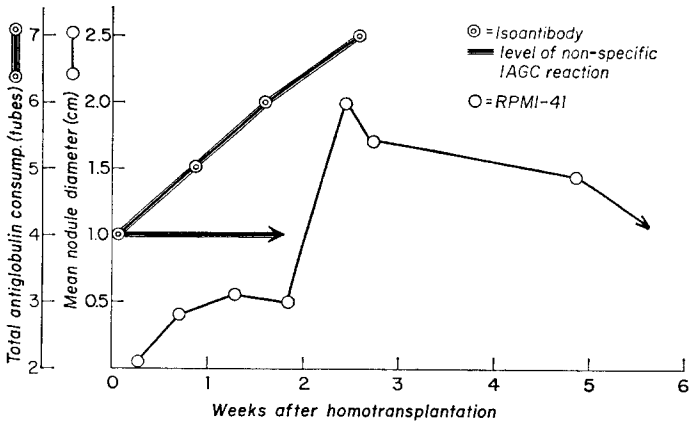

Fig. 5. Isoantibody response and course of growth and rejection of homotransplant nodule following subcutaneous transplantation of RPMI 41 cells in a recipient with advanced cancer of the ovary (No. 25-28-35). Antibody increased progressively during the first 3 weeks. Homotransplant nodule continued to grow during this period but was regressing by 5 weeks and was completely resorbed by 13 weeks.

and regression of the homotransplant nodules, suggesting a causal relationship (Figs. 2 and 3). However, in the cancer-patient recipients the appearance of serum antibody was not promptly or consistently followed by homotransplant rejection (Figs. 4 and 5). These cancer patients produced isoantibody as well as or better than the healthy recipients, but their homotransplants generally grew larger and longer even in the presence of circulating antibody. This lack of relationship was also observed in previous studies with the tanned erythrocyte technique [1, 3].

Comparison with tanned erythrocyte agglutination technique. Several of these same sera, from both normal recipients and recipients with advanced cancer, were studied previously by the tanned erythrocyte agglutination technique [1–3].

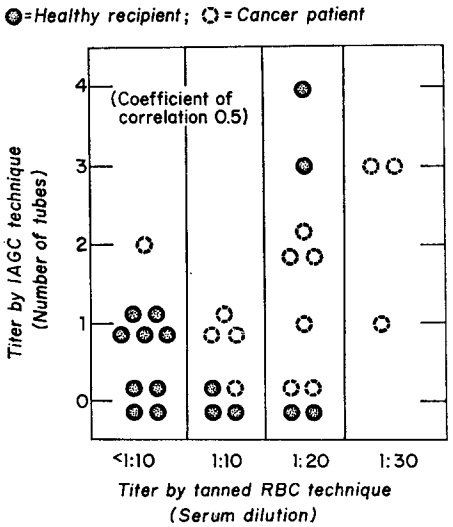

Fig. 6. Scatter diagram comparing isoantibody titers aginst HEp 2 cell antigens as determined by two techniques on individual serum specimens.

Data on thirty sera which were collected 2, 4, and 6 weeks after a primary or repeat homotransplant, and which were studied by both methods with HEp 2 antigen are correlated in Fig. 6. Of the thirty sera, four were negative and twelve were positive by both techniques. Of the remaining fourteen, six were positive by IAGC but negative by tanned erythrocyte agglutination, and eight the reverse. Although nearly half gave discordant results by the two techniques, most of the discrepancies fall within a deviation of ± 1 tube from the line of perfect correlation, and of the eight sera that had IAGC isoantibody titers of two or more tubes, only one failed to show a tanned erythrocyte agglutination titer of 1 : 20 or more.

The two methods of antibody measurement against HEp 2 antigen are also compared on larger but non-identical study groups in Fig. 7. The IAGC data are calculated form Table 2. The antibody curves determined by the tanned erythrocyte technique were calculated from previously published data on eighteen healthy recipients of primary homotransplants (Table 1 of Ref. [2]), and the antibody response to repeat transplants is taken from Fig. 7 of Ref. [1]. Although the study populations are not identical, the results are representative of our total experience and are probably valid for general comparison. From inspection of Figs. 6 and 7 it appears that titers of one, two, or three tubes by the IAGC technique correspond roughly to titers of 1 : 10, 1 : 20, and 1 : 40 by the tanned erythocyte agglutination technique. However, the IAGC technique was less satisfactory for the study of primary antibody responses because of the lower frequency of detectable reactions and because of the poor

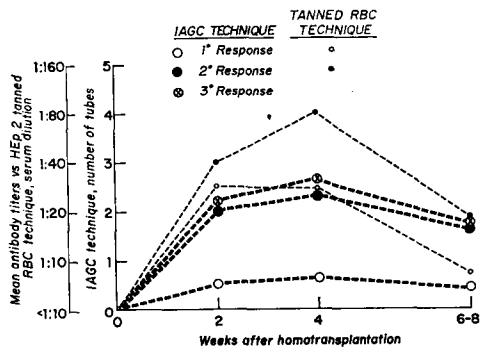

Fig. 7. Comparison of isoantibody responses to repeated homotransplants as measured by two techniques. Averaged data from comparable studies but not from identical serums (see text).

reliability of one-tube IAGC titers. The IAGC technique was quite satisfactory for detection of isoantibody following repeat homotransplants, and it clearly indicated the booster response. Another point of apparent difference between the two techniques was the longer persistence of antibody detectable by the IAGC technique. A possible reason for these slight differences might be the fact that the IAGC technique detects only antibody which reacts with antigens at the cell surface whereas by the tanned erythrocyte technique intracellular as well as cell-surface antigens are available for reaction. It is also possible that the antigens which react in these two techniques differ qualitatively as well as (or instead of) differing in their location in the cell.

DISCUSSION

The studies reported here confirm the previous reports, based on the tanned erythrocyte agglutination technique, that isoantibodies appear in the serum for a few weeks following homotransplantation of tissue-cultured cancer cell lines in human recipients, both healthy men and patients with cancer. The parallelism between results obtained by this technique and the tanned erythrocyte technique is striking in spite of the minor differences. This confirmation by a different serological technique is of interest because our previous attempts to detect these isoantibodies by other methods (including direct agglutination, precipitin, complement fixation and cytotoxic techniques) had not been successful, although perhaps not yet adequately explored. There seems no doubt that it is true isoantibody since it appears during the growth and rejection of the homotransplants, it shows a booster response after repeat homotransplants, and it reacts with human but not with rat cells. These studies do not, and were not designed to, demonstrate whether the antibody is specific for cancer cells or whether it is simply an anti-human species reaction.

The data are clear evidence that the isoantibodies react with antigens at the surface of the tissue-cultured cells. This, however, does not exclude the possibility that the same or different isoantibodies may react with *intra*-cellular components.

The results of these isoantibody studies following repeat homotransplants are much like those reported by Colombani *et al.* following skin grafts [23]. They also resemble the results of Walford *et al.* [24] who found that a serological technique using radioactive labeled antiserum, which is methodologically similar to the IAGC test, was satisfactory for detection of isoantibody only after a second set of skin grafts. These investigators [25] also reported a considerable non-specific adsorption of globulin from pretransplant serum specimens resembling that observed in the present study.

The significance of the *non*-specific reaction is entirely unknown. The increased frequency of this reaction with diseases and/or increasing age is of interest and invites further exploration as an indicator of some type of serum abnormality. It brings to mind other evidences of serum abnormalities in old or ill people, such as rheumatoid factor [26], altered electrophoretic mobility of RBC [27], altered concentrations of serum lipo- and glyco-proteins [28], altered serum electrophoretic patterns [29], and the long known but poorly understood biological false positive reactions with syphilis reagin and acceleration of erythrocyte sedimentation rate. If the non-specific reaction is, even in part, an antigen-antibody type of reaction it raises the possibility that auto-immune reactions are common in cancer, in other chronic diseases, or perhaps in all older people.

The use of a single concentration of antigen (tissue-cultured cells), of test serum, and of antihuman globulin in the first two steps of the procedure was a technical necessity because of the large amounts of tissue-cultured cells required even for the method as used. If the supply of living tissue-cultured cells for use as the antigen were not a limiting factor, the use of serial serum dilutions would have the advantage of reducing the final step to an all or none reaction rather than a titration.

This IAGC technique has the disadvantages of considerable technical complexity, multiple steps at which errors can occur and be succes-

sively multiplied, and crudeness of quantitation. In addition to its inherent shortcomings, the practical usefulness of the technique in its present form is limited because it appears to be less reliable than the tanned erythrocyte agglutination technique for the detection of threshold amounts of antibody, and because some persons cannot be studied by this technique because their sera give a high non-specific reaction.

RÉSUMÉ

Un test indirect de consommation de l'antiglobuline (IAGC) a été utilisé pour la mise en évidence, à la surface de la cellule, d'anticorps réagissant avec des cellules cancéreuses humaines ((HEp2), cultivées "in vitro". Cette technique fut employée pour étudier la formation d'anticorps, à la suite d'homogreffes de différentes lignées de cellules cancéreuses humaines, chez des receveurs sains et chez patients atteints de cancer en phase avancée.

A la suite d'homogreffes primaires, cette technique ne permettait habituellement pas la mise en évidence d'anticorps. Par contre, après des homogreffes répétées, un anticorps fut détecté dans la moitié des cas environ. Chez les receveurs sains, le taux des anticorps approchait du maximum, deux semaines environ après la greffe, atteignait ce maximum après quatre semaines, et diminuait ensuite, pour rester décelable jusqu'à la sixième ou huitième semaine. L'anticorps n'était pas spécifique pour la lignée cellulaire transplantée.

Chez les receveurs sains, les taux d'anticorps variaient parallèlement à la croissance et au rejet du greffon. Cependant, des taux comparables d'isoanticorps apparaissaient chez des sujets cancéreux qui rejetaient le greffon avec un délai plus long, montrant par là que l'anticorps sérique n'est pas une mesure exacte de la capacité de rejet de ces homogreffes.

Les résultats avec le test IAGC sont en accord avec ceux obtenus par la technique de l'agglutination des globules rouges tannés, mais la méthode IAGC n'est pas satisfaisante pour isoler des taux très bas d'anticorps, parce que des modifications s'exprimant par des différences d'une dilution ne sont pas reproductibles.

Les sérums provenant de nombreux sujets, avant transplantation, et particulièrement de patients atteints de cancer ou d'une autre affection altérant l'état général, donnaient une réaction non spécifique qu'il convient de distinguer de l'isoanticorps. La signification de cette réaction non spécifique est inconnue.

SUMMARY

An indirect antiglobulin consumption (IAGC) technique was used for the detection of isoantibody which reacts with live tissue-cultured human cancer cells (HEp 2) at the cell surface. The technique was used to study the development of antibody following homografts of various tissue-cultured human cancer cell lines in healthy recipients and patients with advanced cancer.

Following primary homotransplants, antibody was usually not demonstrable by this technique, but after repeat homotransplants antibody was detected in about half of the recipients. In healthy recipients, antibody levels were near maximum by two weeks after transplantation, maximum by four weeks, and lower but usually still detectable at six and eight weeks. The antibody was not specific for the transplanted cell line.

Although isoantibody levels generally paralleled homograft growth and rejection in normal recipients, comparable levels of isoantibody developed in cancer patients whose rejection of the transplants was impaired — thus indicating that serum antibody is not an accurate measure of ability to reject these homografts.

Results with the IAGC technique are in general agreement with the results obtained with the tanned erythrocyte agglutination technique, but the IAGC method is not satisfactory for very low levels of antibody because "l-tube titers" are not reliable.

Pre-transplantation sera from many persons, particularly patients who had cancer or debilitating diseases other than cancer, gave a non-specific reaction which had to be distinguished from isoantibody. The significance of this non-specific reaction is unkown and unexplored.

ZUSAMMENFASSUNG

Eine indirekte Antiglobulinverbrauch-Methode (IAGV) wurde angewendet, um einen Isoantikörper, der mit lebenden, aus Gewebskulturen stammenden, menschlichen Krebszellen (HEp 2) an der Zellenoberfläche reagiert, aufzudecken. Die Methode wurde bei Untersuchungen über die Entwicklung von Antikörpern nach Ueberimpfung von verschiedenen, in Gewebskulturen gezüchteten, menschlichen Krebszellstämmen auf gesunde Empfänger und auf Patienten mit fortgeschrittenem Krebs angewandt.

Nach primären Homotransplantationen konnte im Allgemeinen dieser Antikörper durch diese Methode nicht demonstriert werden, aber im Anschluss an wiederholte Transplantationen konnte er bei etwa der Hälfte der Empfänger nachgewiesen werden. Bei gesunden Empfängern war der Antikörperspiegel maximal zewi Wochen nach der Transplantation, blieb maximal während 4 Wochen und war zwar niedriger aber immer noch nachweisbar nach sechs und acht Wochen. Der Antikörper war nicht spezifisch für den überpflanzten Zellstamm.

Obwohl der Isoantikörperspiegel im Allgemeinen parallel mit dem Transplantatwachstum und der Regression bei normalen Empfängern ging, konnte bei Krebskranken, ein ähnlicher Spiegel nachgewiesen werden, troztdem in diesen Fällen der Rejektionsmechanismus beeinträchtigt ist. Hieraus kann geschlossen werden, dass der Serumantikörper keinen genauen Masstab für die Rejektionsfähigkeit gegen diese Transplantate darstellt.

Die Resultate der IAGV-Methode stimmen im Allgemeinen mit denun der gegerbten Erythrozytenagglutinationstechnik überein, aber die IAGV-Methode ist nicht zufriedenstellend, wenn nur niedrige Antikörperspiegel vorhanden sind, da "Ein-Röhrchen-Spiegeldifferenzen" nicht reproduzierbar sind.

Prätransplantationsseren von verschiedenen Personen, insbesondere von Krebspatienten oder von Patienten mit anderen debilitierenden Krankheiten, zeigten eine unspezifische Reaktion, welche von dem Isoantikörper unterschieden werden musste. Das Wesen dieser unspezifischen Reaktion ist unbekannt und unerforscht.

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Growth Rate and Radiosensitivity of Human Tumours*—I

Growth Rate of Human Tumours

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INTRODUCTION

IT IS evident that the growth rate of a tumour has a great influence on the life expectancy of its host. In comparison with slowly growing tumours, those with a high growth rate will sooner lead to a state which is incompatible with life. There are indications that the frequency of blood-borne metastases increases as the growth rate of the primary tumour increases. In view of its importance for prognosis, it is remarkable that relatively few investigations have been made with the aim of establishing more exact data concerning the growth rate of human tumours. In 1956, V. P. Collins *et al.* [1] published the results of measurements of pulmonary metastases. These authors measured the increase in diameter of the shadows of pulmonary metastases on roentgenograms. The growth rate, assumed to be constant for malignant tumours, was expressed in terms of the 'doubling time,' i.e. the time necessary for the metastasis to double its volume. The values for these 'doubling times' proved to be different for different types of tumour, ranging from 11 to 164 days in the series of twenty-four patients studied. Estimations of growth rates of pulmonary tumours were published by Nathan *et al.* [2], Garland *et al.* [3] and Spratt *et al.* [4–6]. Growth rates were also established for colon carcinomas by Welin, Youker and Spratt [7, 8], using successive roentgenograms. Most of these investigations suggest an exponential growth pattern for malignant tumours during the period of observation. However, the data in most instances were insufficient to prove a constant growth rate beyond doubt.

In general, primary tumours cannot be observed for any appreciable period without being treated. Moreover, in most instances the irregular form and their location beneath the surface make accurate measurements impossible. Of all the metastases, those in the lungs, if lobular and sharply-defined, offer good possibilities for volume measurements. Therefore it seemed worthwhile to perform very precise measurements on pulmonary metastases, as a means of studying the growth characteristics of malignant tumours during observation.

METHODS

The cases for this study were selected from a group of patients with pulmonary metastases of various types of tumours. They were considered suitable if the shadows on the roentgenograms were lobular and well-defined, and if three or more films, made at sufficiently long intervals, were available. A few cases with only two chest-films were also included, because they provided data on the growth rate of tumours of a particular type. Cases in which the pulmonary metastases showed central cavitation attributable to necrosis were excluded. Careful inspection of the shadows of pulmonary metastases shows that each usually has a margin composed of segments of circles. Frequently, one can detect in the inner part of the shadow opaque areas of different intensities,

*Abbreviated version of thesis (Leiden, April, 1965).

the borders of which are also circular. In many instances the impression is gained that segments of a thin, dark line encircle these spherical parts. This phenomenon may be explained on the basis of the Mach effect, as illustrated in a roentgenogram of plastic spheres in Fig. 1. The impression is that each metastasis usually is made up, like a bunch of grapes, of a number of spherical components. During growth, the shadows of these elements will overlap one another over increasing areas. Assuming a constant growth rate, a number of elements of approximately the same size and close together. will lead to a shape of the total that again is more or less spherical or ovoid (Fig. 2). If the elements originally are very different in size, sooner or later during growth the largest one will overlap all the others (Fig. 3). Thus it is essential to follow each element of a metastasis in all successive roentgenograms if one is to obtain reliable information about the growth rate of a tumour.

For measurement transparent plastic rulers (Fig. 4), in which circles of increasing diameter were engraved, were shifted over the elements of the metastasis on the roentgenograms. It proved possible to select very accurately the circle best fitting around one of these elements their diameter. The increase in the volume as a function of time will have an exponent of three relative to the increase in diameter.

of a metastasis. In most instances the measurements could be reproduced to within 1 mm difference, when dealing with shadows having diameters of ~1–4 cm and with larger tumours within 2 mm. As during virtually all exposures the same focus-film-distance (1·50 m) and position of the patient were used, and since metastases always remain in the same place, no factor for enlargement was introduced in calculating growth rate. The growth curve was obtained by plotting the diameters of the shadows of the metastases against time. As the form of the components of the shadows remains circular in different projections, it can be assumed that the components are spherical, enabling one to calculate their volume from

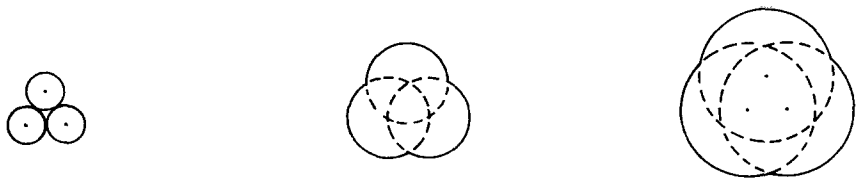

Fig. 2. Overlapping on the roentgenograms of metastases with identical diameter during constant growth.

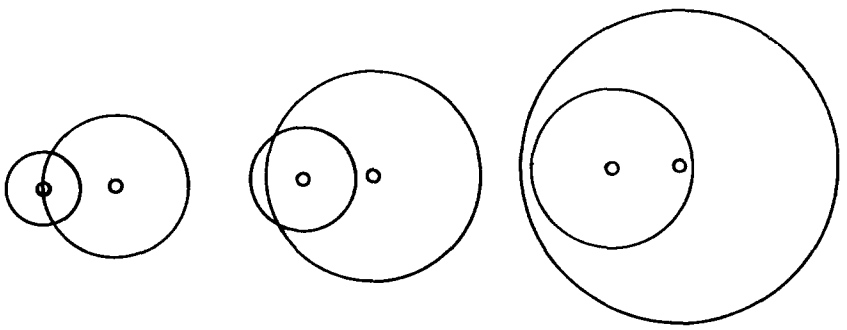

Fig. 3. Overlapping on the roentgenograms of metastases with different diameter during constant growth.

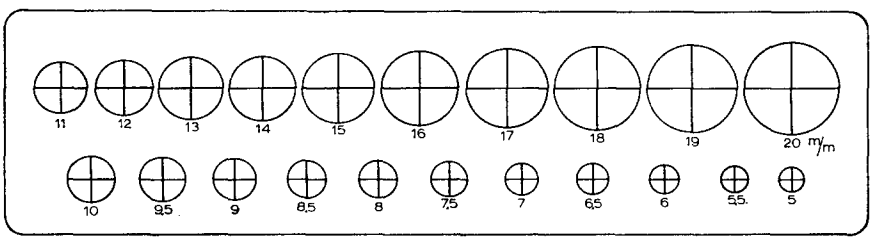

Fig. 4. Transparent plastic ruler with engraved circles.

RESULTS

Measurements of pulmonary metastases were performed on successive roentgenograms of eighty-six patients. In seventy-seven cases the same metastases could be measured at sufficiently long intervals on three or more chest films, with an average of 7·8 films per patient. The

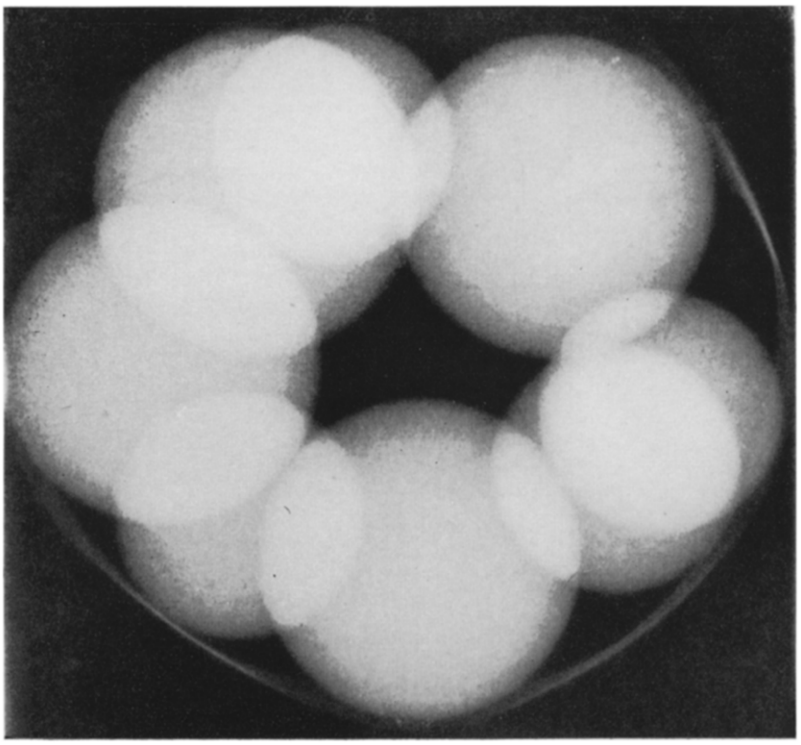

Fig. 1. *Roentgenogram of plastic spheres illustrating the Mach effect.*

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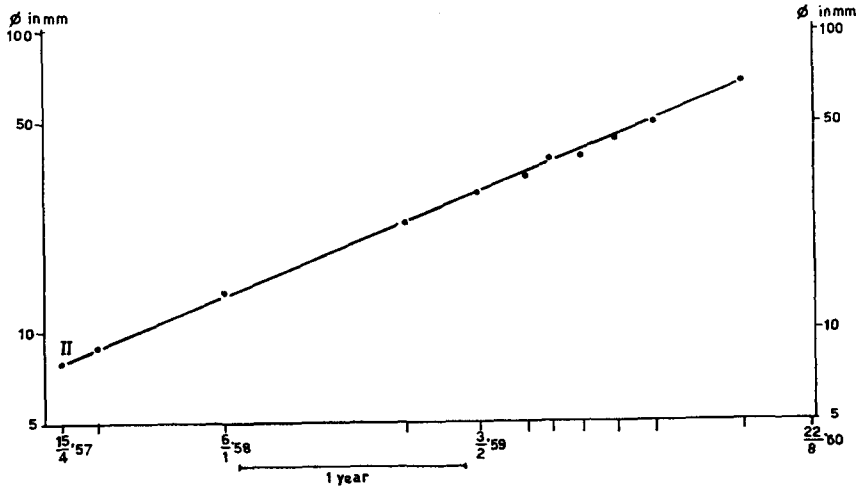

Fig. 5. *Example of a growth curve of a pulmonary metastasis (fibrosarcoma) (Table* 1, *patient no.* 12).

growth curve, obtained by plotting diameters in millimetres against time, showed an exponential form in seventy of the seventy-seven cases. When one plots the logarithms of the diameter, or uses a logarithmic scale the locus of the points very closely approximates a straight line (Fig. 5). This constant growth rate was observed not only with rapidly growing tumours, but also over periods of several years with slowly growing ones. In a selected group of seven cases for each of which ten or more chest-films taken at sufficiently long intervals were available, covering a period without treatment, mathematical analysis showed that the points on the growth curve fitted a model of exponential growth for the metastases. The doubling time T_2, i.e. the time needed by a tumour to double its volume, can be calculated or measured from the curve, for instance by deriving the time interval necessary for the metastases to grow from 10 to 20 mm diameter. One third of this interval comprises the doubling time, since when the diameter has doubled the volume has increased by a factor of 2^3. The value of T_2 derived from the drawn curve did not differ substantially from the value calculated mathematically.

Different pulmonary metastases in one given patient show exactly the same growth rate, even when they differ in size. Only in rare cases is one able to estimate the growth rate of a primary or recurrent tumour, and at the same time or later estimate the growth rate of the pulmonary metastases. We had only one patient in whom measurements of a recurrent fibrosarcoma near the knee joint were performed over a long period, during which, apart from a small excision to free a nerve, no treatment was given. The growth here also (Fig. 6) suggest an exponential pattern with a rate approximately equal to the growth rate of the pulmonary metastases in the same patient (Fig. 5), T_2 being 122 days.

Table 1 comprises the main data on the patients, their primary tumours and the pulmonary metastases. There is a great variation in doubling times, even in each group of cases of similar type and localization.

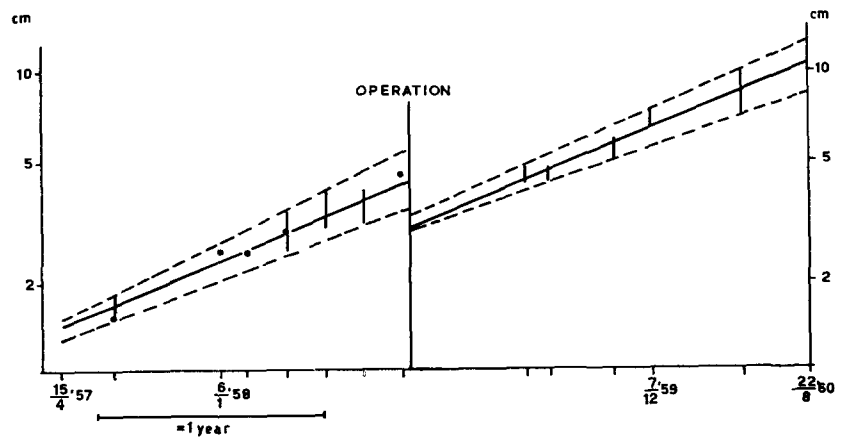

Fig. 6. *Growth curve of a recurrence of a fibrosarcoma near the knee joint (Table* 1, *patient no.* 12).

Table 1. Summary of the examined cases

Patient No.	Sex	Age	Primary tumour: Histological type	Localization	Intervals in months*: First symptom	First metast.	Survival	Doubling-time T_2 in days
1	♀	29	Fibrosarcoma	Groin	− 6	− 36	+ 61	20?
2	♀	53	,,	Axilla	− 6	− 14	+ 22	27?
3	♂	26	,,	Calf	− 6	− 1·5	+ 43	32
4	♂	23	,,	Thigh	−38	− 35	+ 36	33
5	♀	60	,,	Forearm	− 1	− 41	+ 10	52
6	♀	87	,,	Breast	− 2	− 23	+ 6	54
7	♀	18	,,	Shoulder	− 1	− 30	+ 56	67
8	♂	47	,,	Thoracic wall	−12	+ 22	+111	70
9	♂	38	,,	Thigh	− 8	− 55	+ 35	77
10	♀	29	,,	Arm	− 3	− 47?	+ 79	87?
11	♀	26	,,	Hand	−24	+198	+300	97?
12	♂	39	,,	Knee region	− 6	− 96	+ 78	122
13	♂	23	,,	Knee region	−24 or longer	−204	+ 18	212
14	♂	43	,,	Face	0	−180	+ 72	225
15	♂	47	,,	Knee region	−12	+ 24	+216	238
16	♀	70	,,	Groin	− 6	−236	+ 50	257
17	♂	49	Lipo(myxo) sarcoma	Thigh	− 5	+ 35	+ 56	13·5
18	♂	38	Polymorphic cell sarcoma	Calf	?	− 19	+ 20	30
19	♀	21	Malignant synovioma	Thigh	− 0·5	− 13	+ 26	30
20	♂	10	,, ,,	Fibula	− 1	− 28	+ 16	33
21	♂	42	Rhabdomyosarcoma		− 3	− 19	+ 14	35
22	♂	15	Leiomyosarcoma	Cheek	− 1·5	− 31	+ 36	44
23	♂	29	Malignant haemangio-pericytoma	Forearm	−24	− 86	+ 59	115
24	♀	19	Osteosarcoma	Tibia	− 1	− 4	+ 8	17
25	♂	10	,,	Fibula	− 2	− 15	+ 12	17
26	♂	26	,,	Femur	− 2	− 5	+ 9	21
27	♂	13	,,	Femur	− 2	− 23	+ 2	23
28	♀	14	,,	Femur	− 2	− 30	+ 11	25
29	♂	8	,,	Tibia	− 2	− 15?	+ 23	26–53
30	♂	21	,,	Tibia		− 24	+ 11	27
31	♂	12	,,	Femur	− 1	− 36	+ 12	36
32	♀	10	,,	Humerus	− 2	− 35	+ 10	39
33	♂	18	,,	Femur	− 4	− 35	+ 10	43
34	♂	18	,,	Fibula	− 3	− 38	+ 28	55
35	♂	13	,,	Femur	− 2	− 48	+ 22	56
36	♂	9	,,	Fibula	− 2	−252	+ 52	253
37	♂	19	Undifferentiated bone sarcoma	Femur	− 4	− 16	+ 11	16
38	♂	18	Chondromyxoid sarcoma	Fibula	?	− 22	+ 29	38
39	♂	17	Ewing sarcoma	Femur	?	− 80	+ 22	75
40	♀	26	Reticulum cell sarcoma	Neck	− 8	− 16	+ 10	19
41	♀	75	Reticulum cell sarcoma	Maxilla	− 5	−154	+ 28	135
42	♂	34	Embryonal and chorio-carcinoma	Testis	−0·5	+2·5	+ 7	3–4
43	♂	31	Embryonal carcinoma	Testis	− 1	− 2	+ 11	10
44	♂	48	Embryonal carcinoma	Testis	− 3	− 8	+ 6	12·5
45	♂	22	Embryonal carcinoma	Testis	− 6	− 10	+ 6	13
46	♂	36	Embryonal carcinoma	Testis	− 6	− 17	+ 6	16
47	♂	28	Embryonal carcinoma	Testis	− 7	− 16	+ 16	17
48	♂	42	Embryonal carcinoma	Testis	− 1	− 10	+ 18	17
49	♂	36	Embryonal carcinoma	Testis	− 6	− 37	+ 3	32
50	♂	49	Adenocarcinoma (malignant teratoma)	Testis	− 6	−234	+ 41	205
51	♀	59	Adenocarcinoma solidum	Breast	− 1	+ 2	+ 24	23
52	♀	68	Carcinoma solidum	Breast	− 3	− 36	+ 12	39
53	♀	60	Carcinoma	Breast	− 2	− 15	+ 16	52
54	♀	71	Carcinoma solidum	Breast	−12	−144	+ 8	159
55	♀	53	Carcinoma	Breast	− 1	−166	+ 50	177
56	♀	52	Adenocarcinoma	Breast	−90	−804?	+160	745
57	♂	61	Cylindroma	Submandibular region	− 2	− 34	+ 17	37

Table 1.—*continued*

Patient No.	Sex	Age	Primary tumour Histological type	Localization	Intervals in months* First symptom	First metast.	Survival	Doubling-time T_2 in days
58	♂	29	Cylindroma	Nasal cavity	−36 (−192)	−190 (−156)	+ 52 (+76)	200
59	♂	61	Cylindroma	Nasal cavity	−16	−207	+173	307
60	♀	44	Adenocarc.	Parotid region	−36?	−155/ 237	+ 69	188/254
61	♂	73	Undifferentiated carc.	Base of tongue	−12	− 9	+ 16	20
62	♂	59	Squamous cell carcinoma	Lung	− 2	− 24	+ 9	24
63	♂	66	?	Lung?	− 5	− 69	+ 24	57
64	♂	61	Squamous cell carcinoma	Lung	−24 or longer	−107	+ 68	153
65	♀	68	Transition cell carcinoma	Bladder and renal pelvis	− 5	− 8	+ 12	18
66	♂	50	Low diff. carcinoma	Bladder	− 2	+ 3 (− 8)	+ 28	23
67	♀	50	Carcinoma solidum	Bladder	− 1	− 21	+ 12	27
68	♂	57	Squamous cell carcinoma	Bladder	−11	−122	+ 47	125
69	♂	66	Papill. transition cell ca.	Bladder	− 2	− 61	+ 96	135
70	♂	42	Grawitz carc.	Kidney	− 6	+168	+ 11	157
71	♀	38	Squamous cell carcinoma	Uterine cervix	− 5	− 16	+ 30	34
72	♀	51	Squamous cell carcinoma	Uterine cervix	− 9	+ 12	>123	85
73	♀	64	Adenocarc. solidum	Uterine corpus	− 2	− 72	42	83
74	♀	66	Adenocarc. solidum	Uterine corpus	− 6	− 19	+ 31	45
75	♀	67	Adenocarc. solidum	Uterine corpus	− 2	− 71	+ 36	102
76	♀	60	Adenocarc.	Uterine corpus	−2(?)	−456	+197	547
77	♀	77	Adenocarc. solidum	Ovary	−8(?)	− 41	+ 21	51
78	♀	42	Giant cell carcinoma	Thyroid gland	− 2	− 37	+ 4	37
79	♂	72	Carcinoma solidum	Rectum	−13	− 70	+ 15	63
80	♂	41	Adenocarc.	Rectum	?	− 75	+ 88	135
81	♂	31	Malignant melanoma	Skin	− 8	+ 28	+ 67	31
82	♂	44	Malignant melanoma	Skin	− 1?	− 6	+ 38	40
83	♂	65	Malignant melanoma	Skin	− 2	− 67	+ 52	102
84	♀	5	Wilms's tumour	Kidney	− 1	− 11	+ 14	13
85	♀	34	Leiomyo sarcoma	Uterus	− 2	− 8	+ 31	29
86	♀	50	Polymorphic cell sarcoma	Uterus	− 7	− 26	+ 21	35

*The intervals refer to the number of months before (−sign) or after (+sign) the day of the first treatment of the primary tumour.

By arranging the doubling times into groups, it can be shown that doubling times of 10–20, 20–30 and 30–40 days occur most frequently, having been observed respectively in fifteen, eleven and sixteen cases, comprising together nearly half of the total number of cases. Grouping the T_2 values according to a logarithmic system (0–20, 20–40, 40–80 etc.) gives a more equal distribution of frequencies. A comparison of the doubling times for pulmonary metastases from different histological types of primary tumour (Table 2) shows a considerable spread in each group. Apart from embryonal carcinoma of the testis and well differentiated adenocarcinoma, there is an overlapping of the T_2 distribution for all histological types. This means that virtually no diagnostic value as to histological types can be derived from establishing the doubling time of a tumour [9]. Embryonal carcinomas of the testis and osteosarcomas seem to have the smallest range of doubling times, with most of these tumours showing T_2 values of 10–20 and 20–40 days respectively.

Growth rate and mitotic activity

We only had the opportunity to compare the number of mitoses in the primary tumour with the growth rate of the pulmonary metastases in fifteen cases of fibrosarcoma, making use of the study of this type of tumour by van der Werf-Messing and van Unnik [10]. It was not possible to show a correlation of any significance; at most, one could observe that the mean number of mitoses in tumours with a doubling time of 80 days and longer was about half of that in tumours with doubling times of from 20 to 80 days (Table 3).

Table 2. Distribution of doubling times of pulmonary metastases from tumours of different histological type

Histological type	Doubling times T_2 in days					
	0–10	10–20	20–40	40–80	80–160	160–>
Embryonal carc. of the testis	1	6	1			
Osteosarcoma		2	7	3		(1)
Other bone sarcomas		1	1	1		
Fibrosarcoma			4	5	3	4
Other soft tissue sarcomas		1	4	1	1	
Squamouscell carcinoma			3		2	
Transitional cell carcinoma		1	2		2	
Adenocarcinoma (undifferentiated)			3	3	1	
Adenocarcinoma (well differentiated)				1	3	5
Cylindroma			1			2
Remaining tumours		2	4	2	2	
Total	1	13	30	16	14	12

Table 3. Fibrosarcoma. Number of mitoses in primary tumours (ten microscopic fields) compared with doubling times of their pulmonary metastases)

T_2	Number of mitoses	Mean number	
20–40	11, 21, 0, 0	8	8·6
40–80	9, 22, 7, 3, 4	9	
80–160	14, 0, 0	4·7	4·5
160–>	6, 2, 5	4·3	

Influence of age and sex

For the total of eighty-six cases, the spread of the doubling times in the fifty-two men was very similar to that in the thirty-four women, so that no influence of the patient's sex could be detected (Table 4). By studying the distribution of doubling times according to the age of the patient, a statistically significant increase in the T_2 values was shown with increasing age. This holds true both for men and women considered separately, and for the total group of patients. In Table 5, this tendency is apparent, especially for the older age-groups. This means that one cannot expect very long doubling times in young patients, but that although in old patients tumours of the slowly growing type are most frequent, rapidly growing ones may still occur (Table 5).

Table 4. Distribution of doubling times in male and female patients

T_2	Men	Women	Total
0–10	1	0	1
10–20	9	4	13
20–40	17	13	30
40–80	10	6	16
80–160	8	6	14
160–>	7	5	12
Total	52	34	86

Duration of the tumour-process in relation to the doubling times of the metastases

Table 6 presents some data concerning the duration of the tumour-process. There are no differences attributable to sex. As the doubling times increase, the mean time intervals from first symptom to first treatment, first treatment to death, and also from first symptom to death, are seen to increase steadily. Statistical analysis by the Mathematical Centre in Amsterdam proved the relationship to be of the type:

Table 5. Doubling times in different age-groups

Age	Men		Women		Total	
	No. of patients	Mean T_2	No. of patients	Mean T_2	No. of patients	Mean T_2
0–20	12	39	5	32	17	37
20–40	16	50	7	45	23	48
40–60	14	94	8	75	22	87
60–80	8	100	12	122	20	113
Total	50	68	32	79	82	72

Table 6. Intervals in months between the first symptom and the first treatment and between the first treatment and death for different groups of doubling times of pulmonary metastases

T_2	*Men*			*Women*			*Total*		
	1st sympt. 1st treatm.	*1st treatm. death*	*1st sympt. death*	*1st sympt. 1st treatm.*	*1st treatm. death*	*1st sympt. death*	*1st sympt. 1st treatm.*	*1st treatm. death*	*1st sympt. death*
0–10	0·5 (1)*	7 (1)	7·5 (1)				0·5 (1)	7 (1)	7·5 (1)
10–20	4·7 (10)	15·8 (10)	20·5 (10)	3·2 (5)	13·6 (5)	16·8 (5)	4·2 (15)	15·1 (15)	19·2 (15)
20–40	5·8 (13)	20·8 (16)	27·4 (13)	3·2 (12)	24 (12)	27·2 (12)	4·5 (25)	22·5 (28)	27·3 (25)
40–80	5·5 (7)	37·1 (5)	42·3 (5)	3·6 (5)	23·3 (6)	30·2 (5)	4·8 (12)	30·8 (11)	37·7 (10)
80–160	8·5 (5)	61·6 (7)	65·7 (5)	9·2 (6)	96·7 (3)	105·8 (3)	8·8 (11)	82 (10)	86 (8)
160—>	15 (8)	86·5 (8)	101·5 (8)	27 (5)	105·2 (5)	132·2 (3)	19·6 (13)	93·7 (13)	113·3 (13)

*Number of patients between brackets.

$\log S = \alpha + \beta \log T_2$, in which S is the length of survival after the first treatment.

Extrapolation of growth curves

The straight curve obtained by plotting the logarithms of the diameter or the volume against time makes it possible to extrapolate the curve back in time, until the diameter of a single cell is reached (approximately 10–20 μ). Collins calculated in this way that from a single original cell thirty doublings would have to take place in a metastasis in order to form a tumour of 1 cm dia., and that another ten doublings would give a tumour of 10 cm dia. To estimate approximately the date at which a metastasis had a diameter of one cell, one can start from the date on which the diameter was 1 cm, and go backwards in time over a period equal to $30 \times T_2$. Or, one can extend the curve in the diagram until a diameter of 0·01 mm is reached as is shown in Fig. 7.

In Table 1, the column 'first metastasis' gives the intervals in months between the calculated date of dissemination up to the date of the first treatment. The minus sign indicates an interval before the first treatment and the plus sign after the first treatment. In seventy-six of the eighty-six cases, extrapolation indicates that

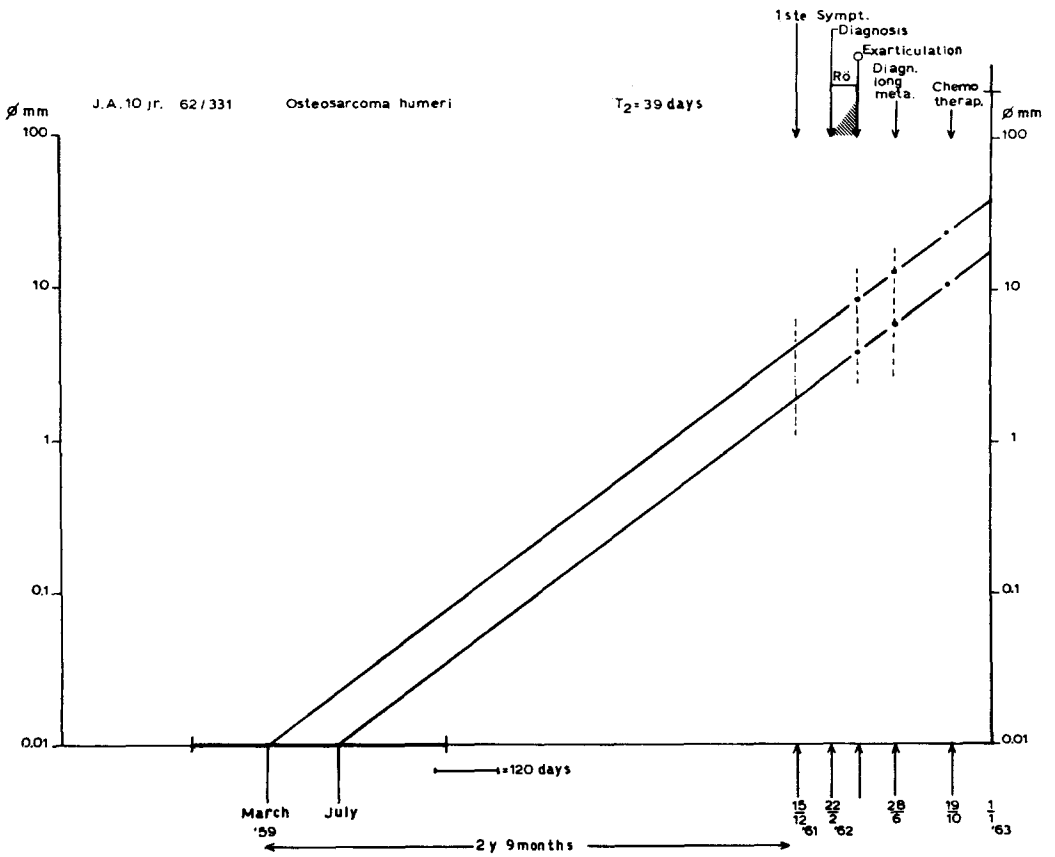

Fig. 7. Extrapolation of a growth curve to a diameter of 10 μ (*Table* 1, *patient no.* 32).

the metastases originated well before treatment of the primary tumour, and in most cases even before the first symptom was observed. Usually, intervals of one year or more were found. Especially when one is dealing with slowly growing metastases extrapolation may indicate very long intervals. Assuming that these patients' primary tumours had a similarly low growth rate, one can see that the primary growth must have existed a long time before the metastases originated. In a small number of cases (Table 1, patients no. 3, 42, 43 and 51) the estimated date of dissemination fell within a few months before or after treatment of the primary. Taking inaccuracies of the method into account, it cannot be excluded that in these cases medical intervention induced the metastases.

Extrapolation in our series of patients never indicated a date of dissemination after the complete removal of the primary tumour, if no metastases outside the lungs were present. This can be demonstrated very clearly in the cases of malignant bone-tumours. In all instances dissemination to the lungs appears to have occurred well before the amputation. The implications on the possibilities of any treatment can easily be seen [11]. Extrapolation in the other direction never pointed to a date on which the total volume of the metastases would have had to exceed the volume of the lungs, with the patient still alive. On the other hand, a number of patients died before a critical volume of the metastases had been reached, an occurrence which could be explained in most cases by the existence of metastases elsewhere in the body.

Atypical growth curves and doubling times

In only seven of the total of eighty-six cases did the growth curve deviate from the exponential type or indicate a doubling time that could not have existed from the beginning of the metastases. In most cases a credible explanation for this could be found in the observation of central necrosis or in the assumption of an endocrine factor.

(a) *Central necrosis.* Patients with pulmonary metastases showing central cavitation were left out of the series. In three of the total number of patients included in Table 1, central cavitation was seen during observation of the metastases; in two this occurred in only a few of the numerous metastases. In patient No. 73 (Table 1) suffering from adenocarcinoma of the uterus, all metastases initially had a similar constant growth rate, but none of them increased in size after reaching a diameter of about 20 mm, while central necrosis at the same time became visible (Fig. 8). Central necrosis was also seen in the metastases of patient No. 77.

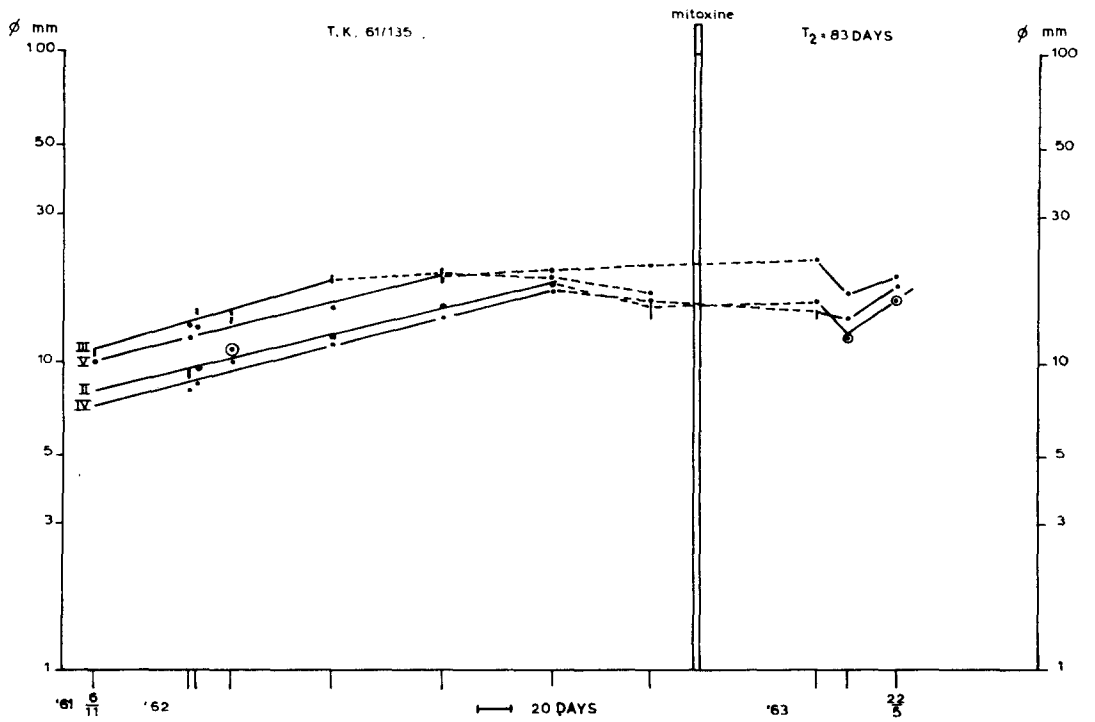

Fig. 8. *Influence of central necrosis on the growth curve of pulmonary metastases (adenocarcinoma of the uterus, patient no.* 73, *Table* 1).

(b) *Retardation of growth by endocrine factors.* In four patients the growth curves suggested a hormonal influence on the natural course of the disease.

Patient No. 56, a woman aged 52, observed a tumour in the right breast in 1943. In November 1950, she consulted the doctor. At that time two sharply defined shadows were visible in the right lung. Palliative irradiation and surgical amputation of the mammary gland were performed. The patient's general condition improved considerably until 1960. The pulmonary metastases were growing very slowly. In July 1961, pleural exudation caused a severe dyspnoea. The patient was treated with cyclosphosphamide (Cytoxan), norandrostenolone phenyl-proprionate (Durabolin) and corticosteroids (Prednisone), with a very favourable effect on the general condition and a rapid decrease in the size of the pulmonary metastases. Though the medical therapy was continued these metastases again started to grow and the patient died in November 1963, i.e. 20 years after the date at which she first observed the tumour.

Figure 9 shows the growth curve in this patient, covering more than 12 years and indicating a doubling time of more than 700 days. Extrapolation in this case would suggest that the metastases had originated many years before the birth of the woman! Since menopause occurred in 1949, 6 years after the first sign of the primary tumour and 1 year before the first treatment, one can assume that menopause and the concomitant lower rate of oestrogen production induced a marked retardation in the growth of the metastases. Perhaps the extremely long doubling time of 547 days for the pulmonary metastases from an adenocarcinoma of the uterine corpus in a 60-year-old patient (No. 76) is the result of a similar hormonal retardation of growth, as extrapolation would suggest an improbable dissemination date corresponding to the patient's fourteenth year of life.

The only case in which a change in growth rate was seen during observation concerns an 9-year-old boy (Fig. 10) with an osteosarcoma of the right tibia irradiated in November, 1955. The growth curves of all the metastases (one of which is presented in Fig. 10) showed a deviation from the exponential shape around the beginning of 1957, suggesting a retardation of growth around his tenth birthday.

Another case is that of a boy aged ten, who had an osteogenic sarcoma. The growth curve of the pulmonary metastases (Fig. 11) illustrates a very low growth rate, with a doubling time of 253 days. Extrapolation, again, would lead to the false conclusion that the metastases had originated 19 years before his birth. It must be assumed that after dissemination a marked retardation in the growth of the metastases occurred in this case. It might be suggested

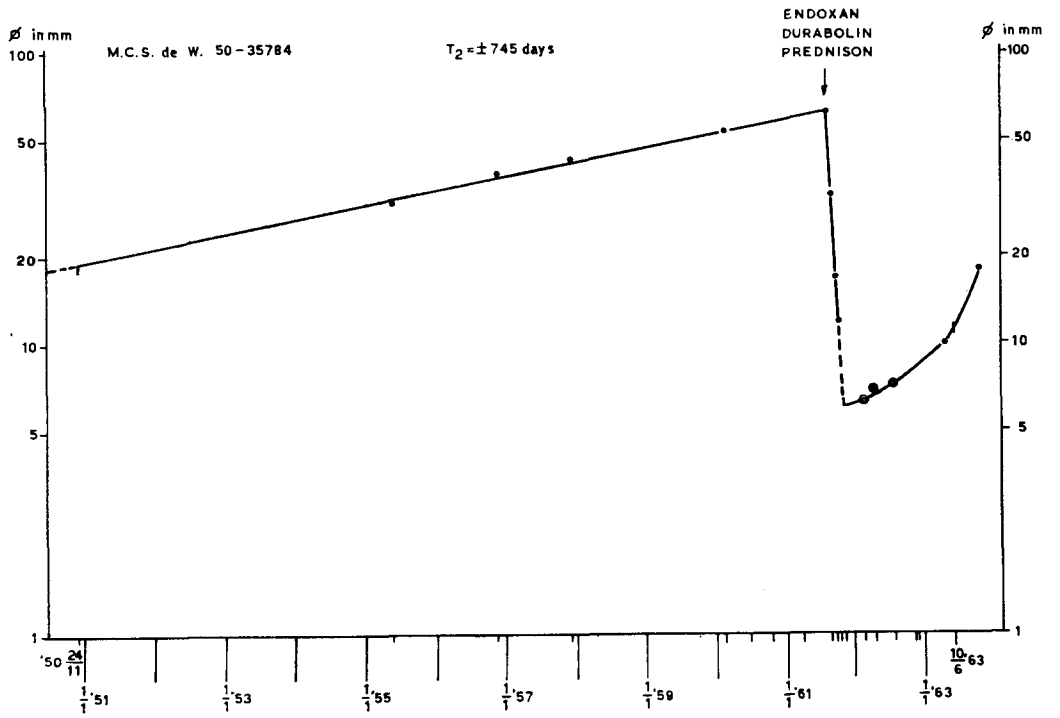

Fig. 9. *Pulmonary metastases with very low growth rate in a patient with breast cancer. Influence of chemotherapy.* (*Table* 1, *patient no.* 56).

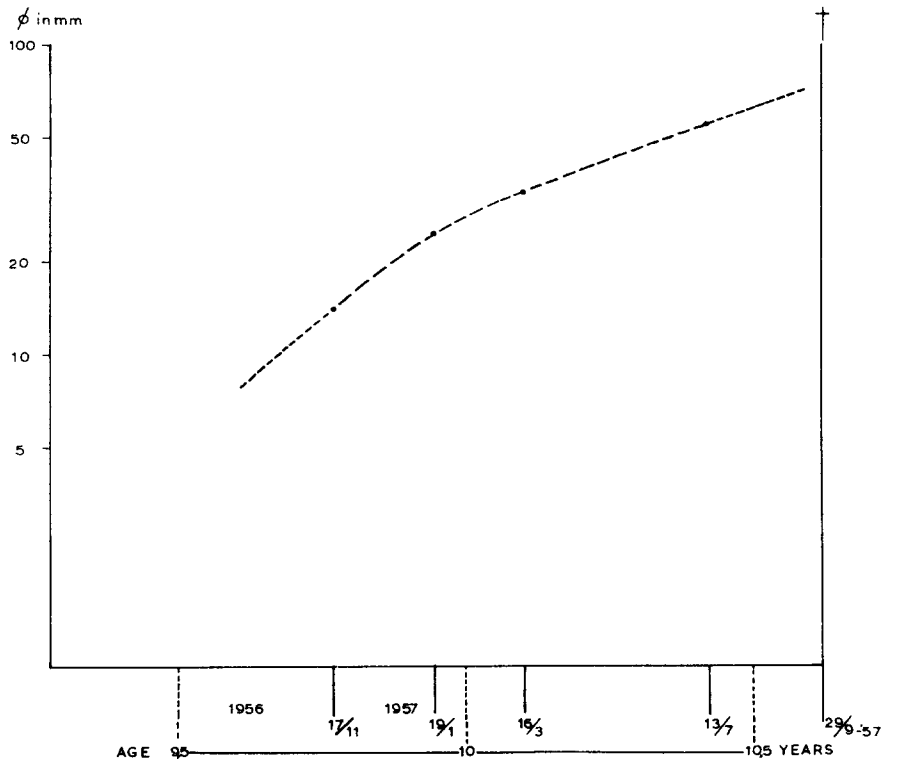

Fig. 10. *Abnormal shape of the growth curve of pulmonary metastasis of osteosarcoma in a 9-year-old boy.*

that the osteosarcoma, similar to that in the patient mentioned before, underwent a change in growth rate when the patient approached the age of 10 years, parallel with the general decrease in the growth rate of children between 9 and 12 years of age [12–14].

(c) *Spontaneous regression of pulmonary metastases.* During the selection of cases for this study, spontaneous regression of pulmonary metastases was seen in two female patients with choriocarcinoma. The outlines of these metastases were too vague to permit precise

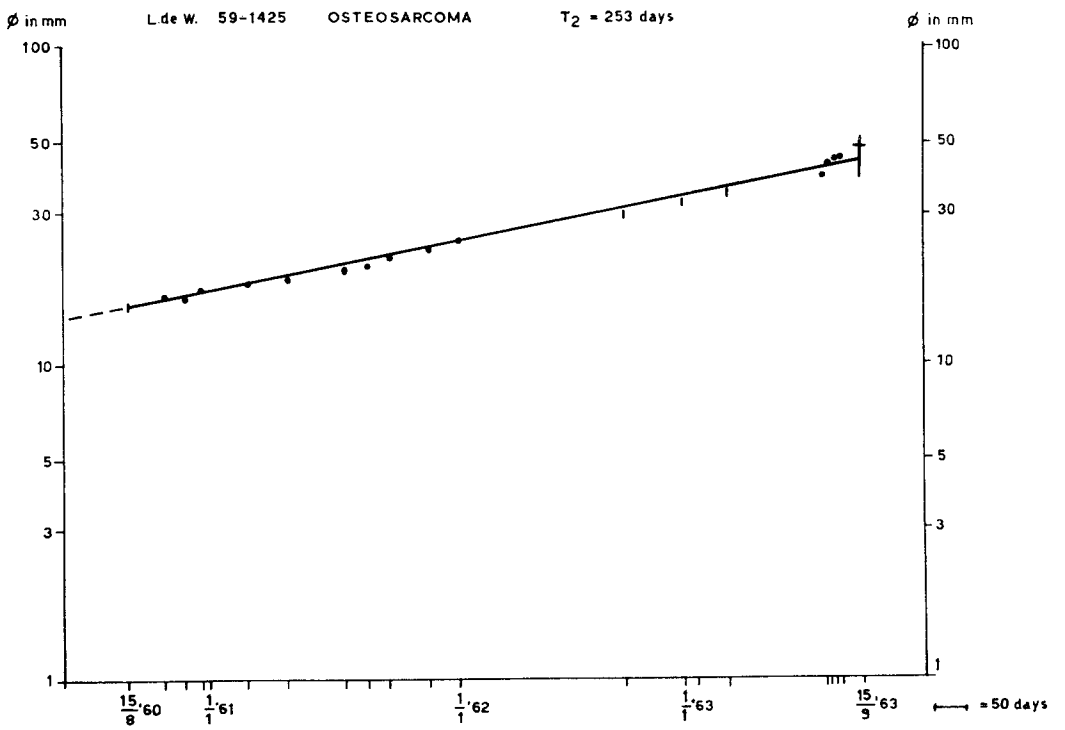

Fig. 11. *Very slow growing pulmonary metastasis in a 10-year-old boy with osteosarcoma.*

measurements, and these metastases were left out of the series. In two male patients suffering from embryonal carcinoma of the testis (Table 1, No. 43 and 48) spotty shadows in the lungs were seen initially, which disappeared afterwards. Later in the course of the disease, more sharply defined, round metastases developed and showed a constant growth rate. With choriocarcinoma in the female, an immunological factor can be assumed to account for regression. In both the male patients, also suffering from a rapidly growing tumour of the reproductive organs, the shadows first seen may have represented inflammatory reactions around tumour-emboli of microscopic size, which themselves only became visible on the chest-films after several months.

Survival times of the diagnosis of pulmonary metastases

The existence of pulmonary metastases can only be demonstrated if these nodules have a diameter of $\sim$ 1 cm. From the growth curve the date on which the metastases had a diameter of 1 cm can be read. The mean values of the survival times from these data show a marked increase with increasing doubling time of the metastases (Table 7).

Table 7. Mean survival time of patients after pulmonary metastases have obtained a diameter of 1 *cm*

T_2	Number of patients	Mean intervals months
0–10	1	(1)
10–20	14	7·4
20–40	29	8·7
40–80	14	14·6
80–160	11	30·5
160–>	10	72·8

So, if the growth rate has been established, one can estimate rather accurately the period of time the patient still has to live.

DISCUSSION

Our observations confirm the hypothesis of Collins *et al.* [1] that most malignant tumours grow according to the formula $V_t = V_0 \cdot e^{\mu t}$ in which V_0 is the original volume, V_t the volume after a given time interval t, e the base of natural logarithms and μ the growth exponent. The growth rates of pulmonary metastases in our patients were of the same order as those given for twenty-four patients by Collins *et al.* There is a marked similarity in growth rates between his largest group, consisting of testicular tumours, and the same group in our material. In eight of a total of nine cases, Collins found doubling times ranging from 9 to 31 days, with a mean value of 22 days. In seven of our nine cases there was a spread of from 10 up to 32 days, with a mean value of 17 days. In one of the two remaining cases there was a T_2 of $3\frac{1}{2}$ days, representing the most rapidly growing metastases we encountered, while in the other, an adenocarcinoma, the doubling time was 205 days. This last case brings to mind the exceptional case in Collins's material, which was a carcinoma with a doubling time of 153 days.

Of course one expects a correlation to exist between growth rate and mitotic activity. We could not demonstrate this in our material by lack of microscopical slides of the pulmonary metastases of which the growth rate was known by measurements. Only a small group of fibrosarcomas offered the chance to compare the mitotic frequencies in the primary tumour with the growth rate of the pulmonary metastases. To arrive at a number of fifteen we had to include a few cases of which only two points of the growth curve could be measured, making the growth rate estimation more dubious. Either this or the unreliability of the method by which the mitotic index was established, can be an explanation for the fact that no clear correlation could be shown.

Though in one case the growth rate of the residual primary tumour appeared to have a similar growth rate as his pulmonary metastasis, one has to consider the possibility that the mitotic rates of pulmonary metastases are different from those in their primary tumours. The investigations of Spratt *et al.* [6–8] dealing with the growth rates of adenocarcinomas of the colon, suggest that the pulmonary metastases may originate from the most rapidly growing areas of the primary tumour.

Most of the studies on experimentally induced tumours in animals suggest that tumours can arise from a very few transplanted cells, which after a short latent period start to grow at a constant rate until a certain size has been reached, after which the growth rate decreases, possibly owing to necrosis [14–24]. The observation in our material, that in nearly all cases a constant growth rate could be demonstrated during the period of observation gives support to the idea that pulmonary metastases grow at a constant rate from the moment of dissemination if extensive necrosis can be excluded. Certainly, there are few or no indications at this moment suggesting another type of growth. Direct

observations of the growth rate in the microscopic phase of a metastasis are not available in Man. Arguments suggesting the improbability of a constant growth rate in the early phases of metastases could be found in cases where extrapolation of the exponential curve pointed to a date of dissemination that was most improbable, e.g. after complete eradication of the primary growth when it can be shown that no extrapulmonary metastases could have been present. In our material there were no such cases found. Only in four cases of the total of eighty-six the date of dissemination obtained by extrapolation fell before the birth of the patient or in early childhood. It could be suggested that the retardation of growth that must be assumed to have taken place during the development of the metastases was caused by hormonal factors. In certain types of primary tumour, a change in clinical malignancy and histological pattern can be observed [25, 26], but probably a rate of constant growth can be assumed to be typical for most primary malignant tumours also [1, 27].

Study of these data makes it clear that the clinician can only observe the ultimate period of any given tumour-process, and that especially with slowly growing tumours, he often is unaware that beginning of the process must be sought far in the past. In general a clinician will estimate growth rate by the increase in volume of a tumour of macroscopical size and by mentally extrapolating in a linear way he will arrive at the assumption that the origin of a tumour lies in a nearby past. For metastases which become apparent only after many years he has to assume tumour cells which remained dormant during a very long period. The exponential growth curve can easily prove there is not any need for such an assumption. Also the latent period from etiological events until the clinical appearance of a tumour can be explained for a great deal by a constant growth rate.

The growth rate is of course of major importance for the prognosis, and especially for the estimation of life-expectancy. Knowledge of the doubling time of pulmonary metastases can provide a guide to treatment. Apart from its significance for estimation of survival times and prognosis, the study of growth rates can give us more insight into the life-history of neoplasms.

RÉSUMÉ

Des mensurations précises, faites sur les clichés radiographiques de 86 *patients, ont montré que les métastases pulmonaires de tumeurs de différents types croissaient à une vitesse constante. Or, on n'observe que quelques exceptions à cette règle. En général, les observations et l'hypothèse de Collins* [1] *ont pu être confirmées. Des métastases pulmonaires différentes, chez un malade donné, avaient une vitesse de croissance semblable, même lorsqu'elles étaient de tailles différentes. Dans un cas, on observa une grande similitude entre la vitesse de croissance de la récidive d'un fibrosarcome et celle de ses métastases pulmonaires. Les taux de croissance variaient largement selon les différents types de tumeurs. Les valeurs moyennes du temps de doublement variaient suivant le type histologique; mais l'écart des valeurs, dans chaque groupe, était tel que le mesure du temps de doublement, dans un cas donné, n'a pas de signification diagnostique. La croissance la plus rapide était observée dans le carcinome embryonnaire du testicule; le fibrosarcome et l'adénocarcinome différencié appartenaient au groupe ou la croissance était la plus lente. Les données étaient insuffisantes pour pouvoir comparer le temps de doublement et l'index mitotique. Dans* 15 *cas de fibrosarcome où le nombre de mitoses dans* 10 *champs microscopiquis* (450×) *fut.déterminé, on ne put mettre en évidence aucune corrélation significative. Tout ce que l'on peut dire, c'est qu'en général les index mitotiques bas furent observés dans les tumeurs à croissance lente, plutôt que dans les tumeurs à croissance rapide. Il n'y avait pas de relation entre les valeurs, et l'écart du temps de doublement et le sexe. Les valeurs moyennes du temps de doublement augmentaient avec l'age. Il est évident qu'on ne peut pas s'attendree à des croissances lentes chez les enfants. Si les tumeurs à croissance lente prédominaient chez les patients âgés, on observait cependant aussi, dans ce groupe, des tumeurs à évolution rapide.*

L'extrapolation des courbes de croissance a également été étudiée. La durée d'évolution des métastases pulmonaires, calculée par cette méthode, apparut beaucoup plus longue que celle estimée dans l'observation clinique des malades. Dans un petit groupe de patients, les mensurations conduisent à établir une courbe que n'est pas compatible avec une croissance

exponentielle, ou amènent à des conclusions absurdes si la courbe est extrapolée. La régression spontanée, la nécrose centrale et l'influence des hormones sont discutées.

La mesure de la vitesse de croissance semble être d'une grande valeur lorsqu'on désire établir un pronostic de survie chez un malade.

SUMMARY

Precise measurements on roentgenograms of 86 *patients showed that pulmonary metastases from different kinds of primary tumour grew at a constant rate. Only a few exceptions to this rule were found. On the whole, the observations and hypothesis of Collins* et al. [1] *could be confirmed. Different pulmonary metastases in any given individual patient were found to have identical rates of growth, even when they differed in size. In one patient, marked similarity was observed between the growth rate of a recurrence of a fibrosarcoma and that of the pulmonary metastases. Wide variations between the growth rates of metastases were found among patients suffering from different types of tumour. Comparison of groups of patients selected according to the histological type of the primary tumour justifies the conclusion that, although marked differences exist among the mean values of doubling times between these groups, the spread of the growth rates within each group is so large that little or no diagnostic value as regards the type of tumour can be derived from measuring the doubling time. Embryonal carcinoma of the testis constituted the category of tumour with the highest growth rate; fibrosarcoma and well-differentiated adenocarcinoma belonged to the slowest growing types. Insufficient data were available for comparing doubling times with mitotic rate. In* 15 *cases of fibrosarcoma in which the number of mitoses per* 10 *microscopic fields* (450×) *was determined, no significant correlation could be demonstrated, although in general lower mitotic frequencies were found for slowly growing as compared with rapidly growing tumours. No difference between sexes was observed as regards the spread of doubling times. Mean values of doubling times for the different age groups showed an increase in the values with increasing age. It is evident that in children long doubling times cannot be expected. Although in old patients slowly growing tumours predominated, rapidly growing types were also seen.*

In addition to these general conclusions, the justifiability of extrapolating the growth curve and the conclusions which can be drawn from this practice are discussed. The total period of existence of pulmonary metastases, as derived from extrapolation of the growth curve, appears to be considerably longer than that estimated from clinical observation of the patient. A small group of patients in whom measurements of pulmonary metastases led to the construction of a growth curve which was incompatible with exponential growth, or in whom this led to absurd conclusions when the curve was extrapolated, is mentioned separately. Spontaneous regression, central necrosis and the influences of hormones are discussed. Measuring the growth rate of a tumour would seem to be of major importance when one wishes to estimate the life expectancy of the patient.

ZUSAMMENFASSUNG

Die genauen Ausmessungen der Rö-Aufnahmen von 86 *Patienten ergaben, dass die Lungenmetastasen verschiedener Primärtumoren jeweils eine Konstante Wachstumsgeschwindigkeit zeigten. Nur wenige Ausnahmen zu dieser Regel wurden entdeckt. Im Allgemeinen wurden die Hypothese und die Beobachtungen von Collins* et al. [1] *bestätigt. Bei den einzelnen Patienten wiesen die verschiedenen Lungenmetastasen eine gliehe Wachstumsgeschwindigkeit auf, selbst wenn sie unterschiedlicher Grösse waren. Bei einem Patienten wurde eine gleiche Wachstumsgeschwindigkeit sowohl des Rezidives des Fibrosarkoms als auch der Lungenmetastasen festgestellt. Bedeutende Variationen wurden gefunden zwischen den Wachstumsgeschwindigkeiten der Metastasen verschiedenen Ursprungs. Der Vergleich von Patientengruppen, deren Metastasen denselben histologischen Ursprung hatten, lässt den Schluss zu, dass trotz grossen Unterschieden in der Verdopplungszeit der Metastasen zwischen den einzelnen Patientengruppen, die Wachstumsgeschwindigkeit innerhalb der einzelnen Gruppen derart unterschiedlich ist, damit aus der Bestimmung der Verdopplungszeit keine oder nur wenige diagnostische Folgerungen über den histologischen Tumortyp gezogen werden können.*

Das embryonale Hodenkarzinom war die Geschwulst mit der grössten Wachstumsgeschwindigkeit. Fibrosarkome und gut differenzierte Adenokarzinome gehören zu den am langsamsten wachsenden Typen. Die Unterlagen genügten nicht, um Verdopplungszeiten mit der Zellteilungsgeschwindigkeit zu vergleichen. Bei 15 *Fällen von Fibrosarkomen wurde die Anzahl der Mitosen pro* 10 *Mikroskopgesichtsfelder* (450×) *bestimmt, aber die Korrelationen waren nicht signifikant, obwohl die Mitosenhäufigkeit bei langsam wachsenden Tumoren geringer als bei schnell wachsenden zu sein schien. Es wurde kein geschlechtsgebundener Unterschied zwischen den Verdopplungszeiten gefunden. Die Durchschnittswerte der Verdopplungszeiten in den verschiedenen Altersgruppen zeigten eine Zunahme mit ansteigendem Alter. Selbstverständlich können bei Kindern keine langen Verdopplungszeiten erwartet werden. Obwohl bei älteren Patienten die langsam wachsenden Tumoren vorherrschten, wurden auch dort schnellwachsende Typen beobachtet. Abgesehen von diesen allgemeinen Schlussfolgerungen, diskutieren die Verfasser die Berechtigung der Extrapolation der Wachstumskurve und die Schlüsse, welche hieraus gezogen werden können. Das Bestehen der Lungenmetastasen, errechnet aus der Extrapolation der Wachstumskurven, scheint bedeutend länger zu sein als man dies aus der klinischen Beobachtung der Patienten schliessen könnte. Die Verfasser erwähnen separat eine kleine Gruppe von Patienten, bei denen das Ausmessen der Lungenmetastasen zu der Konstruktion von Wachstumskurven, welche mit exponentiellem Wachstum unvereinbar sind, führte. Ausserdem wurden die Spontanregression, die zentrale Nekrose und der Einfluss von Hormonen besprochen. Die Geschwindigkeit des Wachstums eines Tumors ist für die Schätzung der Ueberlebenszeit eines Patienten von Bedeutung.*

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Growth Rate and Radiosensitivity of Human Tumours—II

Radiosensitivity of Human Tumours

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INTRODUCTION

THE POSSIBILITY of rather accurately estimating the diameters of pulmonary metastases, and, thanks to their spherical form, also their volumes (see Part I, p. 157, this issue), offered us a chance to observe changes in human tumours after exposure to ionizing radiation.

Our views regarding the effects of radiation on living tissues have been greatly influenced by the results of radiobiological experiments during the past decade. Especially new cell-culture techniques, as originated by Puck and Marcus [1–5] have offered excellent opportunities for studying dose–effect relationships in mammalian cells. Also, *in vivo* experiments carried out in animals have provided a great deal of information [6–9]. Briefly, it can be stated that after a colony of cells has been irradiated, a certain proportion of these cells is left incapable of reproduction, while the remainder after a short mitotic delay start to divide again at a growth rate quite similar to the initial one. Using the ability to proliferate unlimitedly as a criterium for cell survival, the percentage of survivals after exposure to different single doses of irradiation could be established. The dose-survival curves showed a typical form in nearly all experiments (see curve 1, Fig. 1). After a 'shoulder' in the lower dose region, there is an approximately exponential decrease in the proportion of surviving cells as the dose in-increases, giving an almost straight line on a log-scale. According to Barendsen [10], the curve does not become a straight line, but retains a tendency to bend downwards. The added dose giving a 37 per cent 'survival' in the straighter part of the curve serves as an indicator of radio sensitivity. The values of this D_{37} (or D_0) appear to be rather similar in all experiments, independent of the type of mammalian cells used, usually remaining within the relatively narrow limits of 100–200 rads. Under anoxic conditions sensitivity could decrease by a factor up to three. The survival curves can be explained by the target theory [11], assuming that two or more 'hits' are necessary to prevent a mammalian cell from regenerating. Barendsen e.g. [10, 12] studied the effects of radiations having different Linear Energy Transfers, and found that the radiobiological effect decreased with a decreasing LET. The rather uniform reactions of cell colonies in experiments contrast with the great differences in sensitivity among various normal tissues and tumours encountered by the radiotherapist, who finds it difficult to explain his experience on the basis of the oxygen effect alone [13–21]. Elkind *et al.* [22–26] examined the influence of fractionated irradiation, and brought evidence for a virtually complete recovery from radiation injury in the surviving fraction after a relatively short interval. Repeating the same dose after equal intervals would result in an exponential decrease in the number of cells (curve F, Fig. 1).

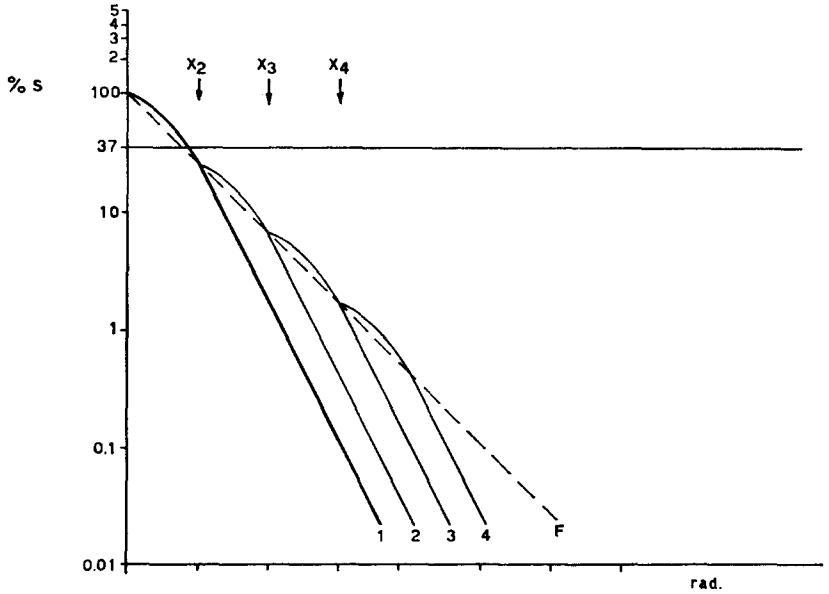

Fig. 1. *Survival curves for fractionated irradiation according to M. M. Elkind (schematic).*

Estimation of the percentage of cells capable of unlimited growth after irradiation is of course not only of interest to radiobiologists, but is also of basic significance for the radiotherapy of malignant tumours. Only a dose which prevents even a single tumour cell from surviving can result in a local cure. So far, this dose can only be chosen roughly on the basis of mean values obtained by studying results in groups of patients with tumours of comparable type, localization and extent. The wide spread in sensitivity observed among individual cases cannot be taken into account.

The radiosensitivities of human tissues and tumours are usually described roughly by the mean effect observed in groups of patients in comparison with for instance skin-reaction [27]. To my knowledge, more precise data are lacking in the literature. Our observations on the growth rate of pulmonary metastases provided a method by which radiosensitivity could be evaluated in patients. The results of a study based on this method is the subject of this report.

METHODS

Of the eighty-six patients (see part I, Table 1) for whom the growth rate of pulmonary metastases was derived from the exponential growth curve, only a few received palliative irradiation to these metastases. In sixteen cases, enough roentgenograms of the lungs were available, covering a sufficiently long period, to enable us to study the effects of these treatments. Roentgenradiation with H.V.L. of 1·5–2·7 mm Cu, and telecobalt radiation were administered. In almost all cases an anterior and posterior portal was used. The doses administered to the metastases were calculated as accurately as possible. No correction was made for the presence of air in the lungs. According to Jacobson and Knauer [28], the inaccuracy introduced by this factor usually can be estimated to be under 10 per cent. Moreover, the error will be in the same direction in all patients. The figures are expressed in rads, using the approximation 1R = 0·96 rads in all cases. For the RBE of telecobalt, a factor of 0·85 was chosen. When any doubt was present concerning the data presented in the record, a recalculation of tumour dose, using lateral roentgenograms of the patient, was performed by the physicist, Drs. A. Somerwil, and the radiologist, Miss H. A. van Peperzeel. The dose rate for roentgen ranged between 75 and 125 R p/min, and for telecobalt, between 40 and 70 R/min. Up to the end of 1962, irradiation was given 6 days a week; later this was changed to 5 days. The daily tumour dose varied, depending on the size of the field and the general condition of the patient, with mean values, calculated over the total period of irradiation, of from 70 to 150 rads. The total tumour dose varied from 1000 up to 6000 rads. Diameters of the pulmonary metastases were plotted on a log-scale against time on a linear scale, before, during and after the course of radiation. The technique of measurements is described in part I.

RESULTS

Influence of radiation on the growth curve

In all cases suitable for measurements during the series of radiation treatments, it could be shown that the individual spherical components of the pulmonary metastases became reduced in size concentrically, either directly or at least within a few days after the beginning of the series of treatments. The borders of the shadows remained circular and rather well-defined and the diameters measurable. The influence of radiation on the growth curve can be demonstrated by the example shown in Fig. 2. It seemed admissible to extend the growth curve to include the period between the date of the last roentgenogram before treatment and the first day of treatment. It was found that when approximately equal daily doses are applied, the diameter (and, assuming a spherical form, also the volume) shows an exponential reduction in size during the course of the treatment. After only a few days the metastases start to grow again at almost the same rate as before the treatment. This was seen in all cases studied. If two metastases of different size in the same patient are irradiated at the same time and with the same dose, their volumes are reduced by the same percentage, so that on a log-scale the curves keep a parallel course before, during and after treatment (Fig. 3).

If in one given patient a group of metastases in one lung is irradiated and groups in the other lung are left untreated, the former group will show the typical regression in diameter, while the others will continue to grow undisturbed (Fig. 4). When the tumour is of a very radiosensitive type, metastases situated near the primary beam may show a temporary standstill in growth or even a small reduction in size, obviously an effect of scattered radiation. If after one course of radiation treatments the tumours have resumed growth at a constant rate and another series is applied to the same group of metastases, a similar reduction is achieved, again followed by resumed constant growth after the last irradiation (Fig. 5).

Pulmonary fibrosis may hamper one's attempts to perform precise measurements after repeated series, if fairly high doses have been given. Another difficulty arises if a metastasis has been reduced to a diameter of less than 5–10 mm. Since it becomes impossible to obtain reliable measurements in this range, one must await regrowth of the metastasis. The total regression in diameter can be estimated by

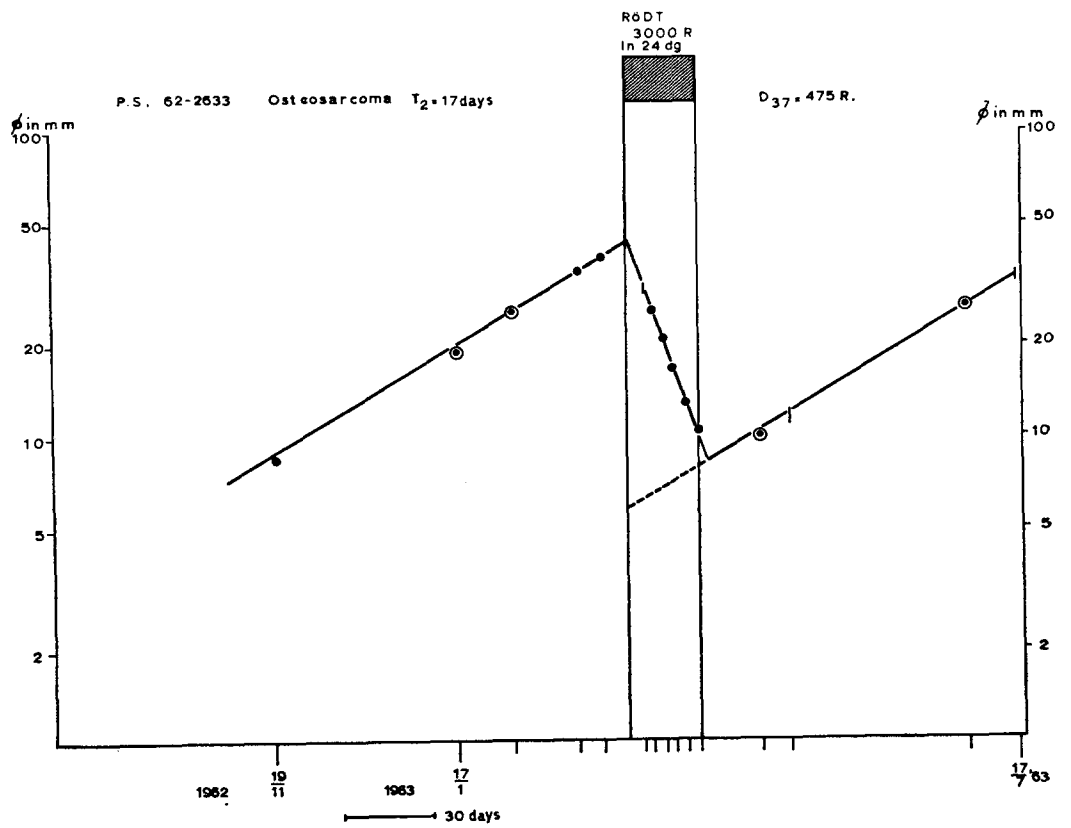

Fig. 2. Effect of fractionated roentgen irradiation on the diameter of pulmonary metastases. (Table 1, patient no. 4).

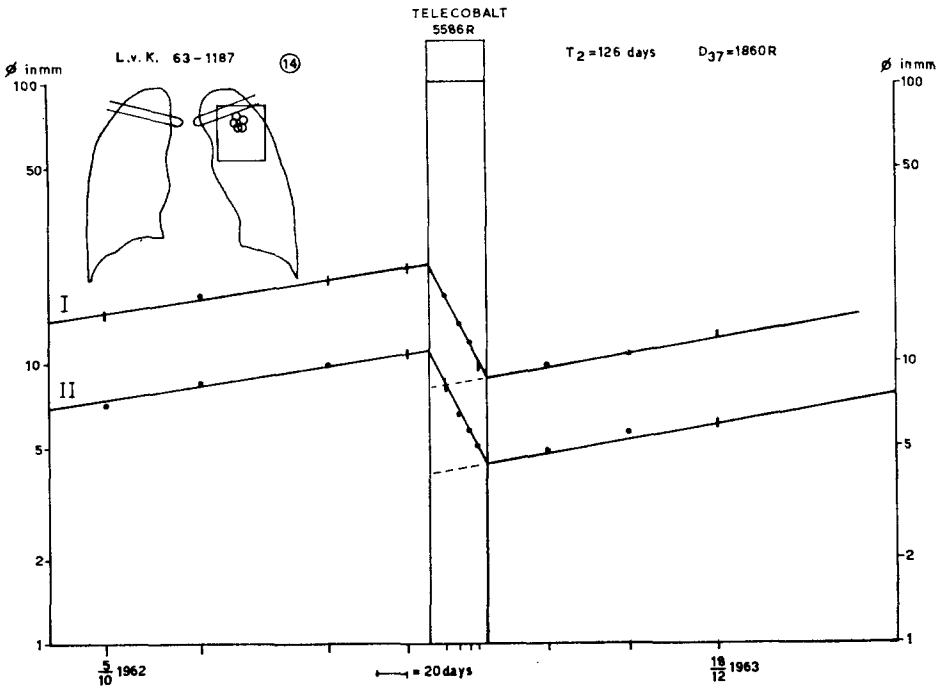

Fig. 3. Effect of telecobalt irradiation on pulmonary metastases with different diameter (bladder carcinoma). (Table 1. patient no. 14).

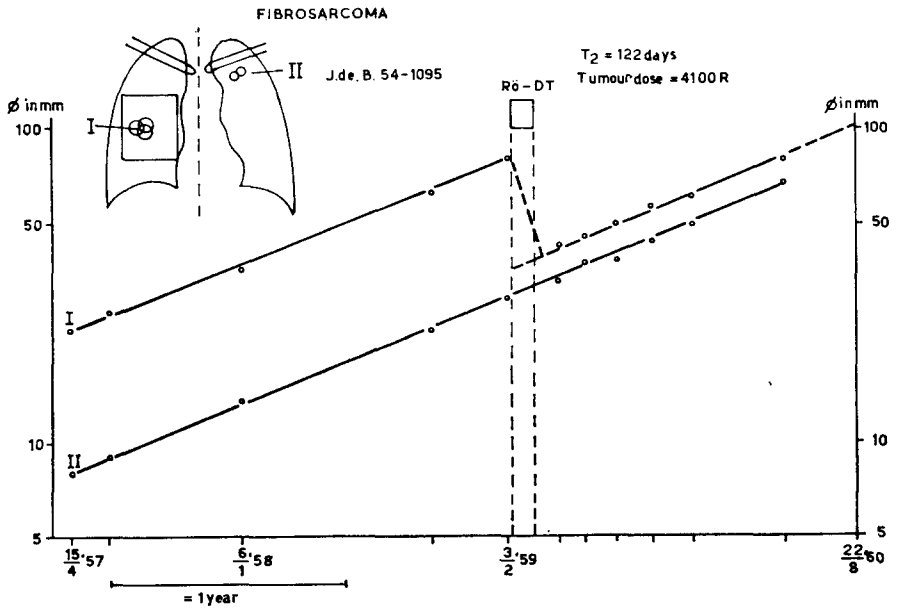

Fig. 4. Growth curves of two groups of pulmonary metastases in one patient, of which only one group was irradiated. (Table 1, patient no. 13).

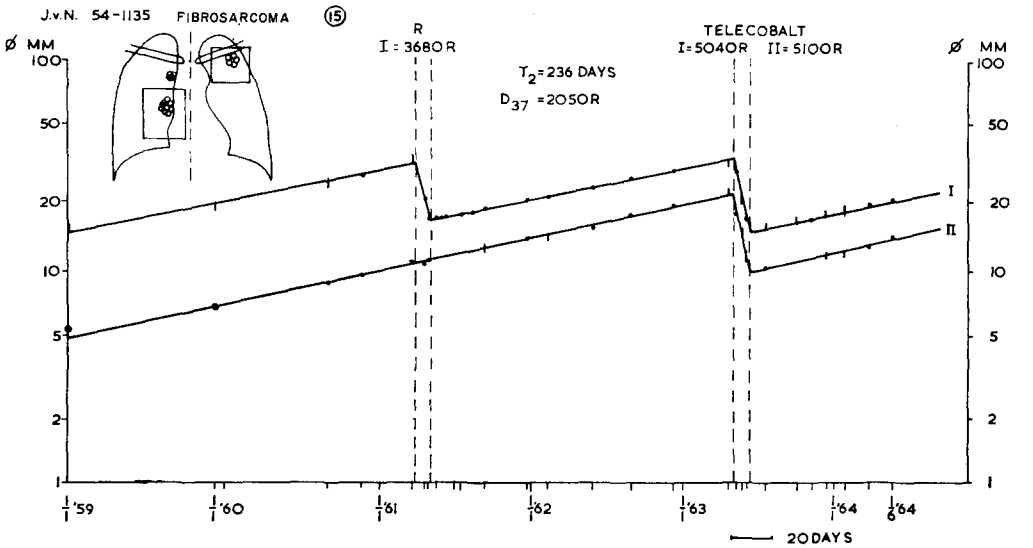

Fig. 5. *Influence of a second series of irradiation with a higher daily and total dose on the same group of pulmonary metastases.*
(*Table* 1, *patient no.* 15).

extrapolating the curve back to the last day of treatment. Several cases illustrate that this method is admissible, as the growth curve before and after treatment nicely joins the curve during the series around the first and last days of the treatment, respectively.

Influence of dose

In several cases it could be demonstrated that, when different metastases in a single patient received the same daily dose, the rate of exponential regression was the same for all metastases, and that the same total dose gave the same percentages of reduction in diameter, as is shown in Fig. 5. In the same diagram one can observe that smaller daily and total doses resulted in a more gradual slope of the curve and a smaller total reduction. The influence of the daily dose could be illustrated very strikingly in the curve of the case presented in Fig. 6.

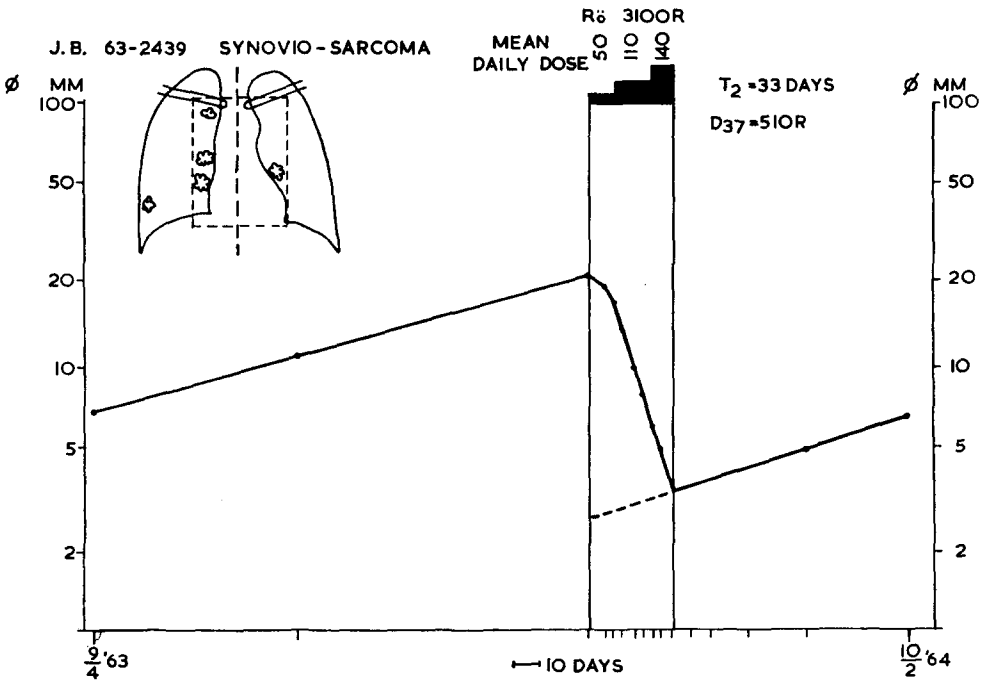

Fig. 6. *Influence of the mean daily dose during fractionated irradiation of pulmonary metastases.*
(*Table* 1, *patient no.* 8).

Estimation of the percentage survival and D_{37}

The demonstrated form of the growth curve and the measurable reduction in the diameter of the tumour provide a possibility of approximating survival percentages in individual cases. The percentage of the original volume of a spherical metastasis remaining after radiation treatment can be calculated by the formula:

$$\frac{d_1^3}{d_2^3} .100,$$

in which d_1 is the diameter just before the beginning of the radiation series, and d_2 that at the end of treatment. When one calculates in this way one disregards any influence of mitoses completed during treatment. It would therefore seem better to extrapolate the growth curve after the series of treatments back to the first day of treatment, and use the diameter shown by the curve for that date as d_2 (see Fig. 2). It must be stated that any mitotic delay induced by the irradiation, estimated in experiments to amount to about one hour for 100 rads, is treated as if it were negligible, as there is a mean interval between treatment sessions of at least 30 hr in the type of fractionation practised [29]. In Table 1 the percentages of "survival" calculated in this way are presented.

In three cases it was possible to calculate for each patient the percentage of survival after two or three separate series of irradiation with different total doses. Assuming that the form of the survival curve for fractionated irradiation as suggested by Elkind also applies to human tumours *in vivo*, one can expect a straight curves starting at 100 per cent with dose 0, if the logarithms of the percentages of survival are plotted against total doses applied. Though the values were obtained in three patients (Figs. 7 and 8) they are in themselves far from conclusive, but they do suggest that the hypothesis may be valid in these cases. As long as there are no indications of another kind of correlation, it seems within reason to assume that the same relationship applies to the other cases, in which only one percentage of survival, for one total dose, is known (Fig. 9). From the curve, the dose leaving 37 per cent of the original volume capable of resuming exponential growth, can be read. If S_D is defined as the percentage of survival after a dose D, the formula

$$D_{37} = \frac{0{\cdot}4318\ D}{2-\log S_D}$$

makes it possible to calculate the 37 per cent survival dose. As can be seen from Table 1, our results showed wide variations in radiosensitivity amongst tumours of different types. Values of D_{37} extending from 300 up to 2550 rads were encountered. These differences, which are equivalent to a factor of 8·5, cannot

Table 1. *Data on sixteen patients of which survival percentages could be calculated. Numbers between brackets refer to Table* 1, *part I.*

Patient no.		Diagnosis	T_2	Tumour dose (R)	Roentgen or Telecobalt	Mean daily dose (R)	%S	D_{37} in rads (RBE=1)
1	(45)	Embryonal carc. testis	12	1225	R	72	2·0	299
2	(44)	Embryonal carc. testis	12·5	1500	R	42	1·2	324
3	(48)	Embryonal carc. with seminoma testis	17	2300	R		0·39	396
4	(25)	Osteosarcoma	17	3000	R	125	0·22–0·25	473
5	(40)	Reticulum cell sarcoma	19	4000	T	131	0·1	470
6	(49)	Embryonal carc. testis	32	2200	R	100	1·7–2·0	504
7	(3)	Soft tissue sarcoma	32	2280	R	62	4·3	691
8	(20)	Synovial sarcoma	33	3150	R	99	0·18	492
9	(57)	Cylindroma (submandib.)	37	2100	T	131	2·3–3·0	506
				3100	T	130	0·94	
				3200–3400	R	±100	0·41	
10		Hodgkin's disease	43	2100	T	131	3·3–4·9	534
11	(77)	Carcinoma of ovary	51	1088	R	100	37	1045
12	(72)	Carc. of cervix uteri	85	6000	T	150	1·4	1142
13	(12)	Fibrosarcoma	122	4100	T	126	7·8–9·9	1369
14	(68)	Carcinoma of bladder	126	5580	T	133	5·2–5·6	1550
15	(15)	Fibrosarcoma	240	3680	R	94	16·4	1974
				5040	T	142	9	
				5100	T	146	8·2	
16	(59)	Carc. nasal cavity	307	4000	R	114	22–23	2558

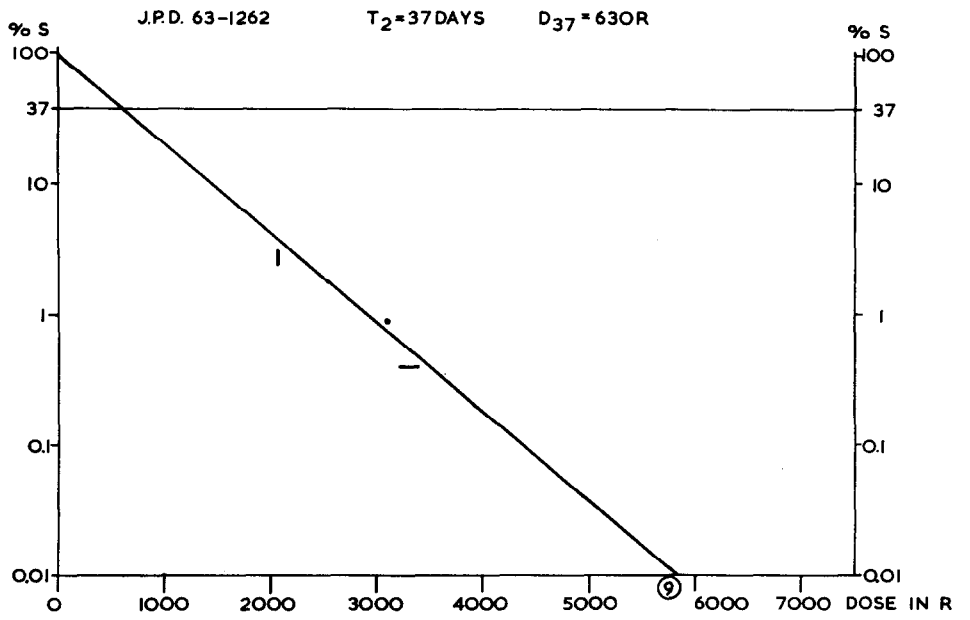

Fig. 7. "Survival" percentages (residual volumes) of pulmonary metastases in one patient after three series of irradiation with different total doses.
(Table 1, patient no. 9).

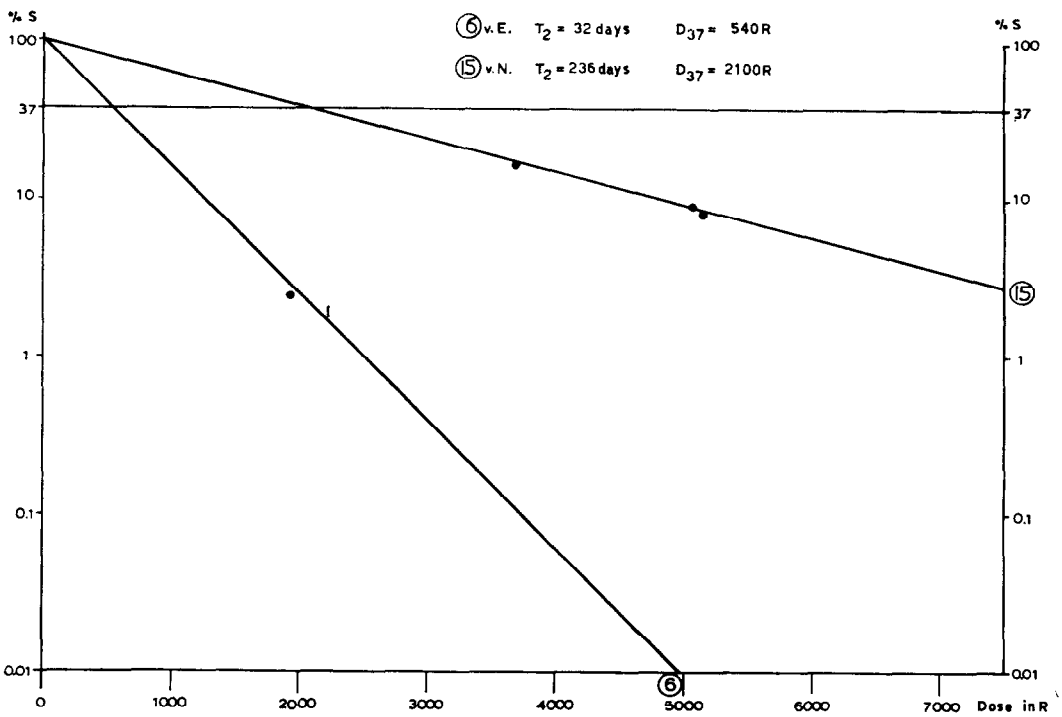

Fig. 8. "Survival" percentages in two patients in which pulmonary metastases were irradiated with series of different total doses.

be explained by the assumption that all cells have the same intrinsic radiosensitivity and that all differences observed are caused by differences in oxygen tension in the tissue. The oxygen effect could only account for a factor of three.

Relation between radiosensitivity (D_{37}) *and doubling time* (T_2)

Though only sixteen cases provided sufficiently reliable data to establish the 37 per cent survival dose (D_{37}), it can be seen in Table 1 that low values were obtained in cases with a

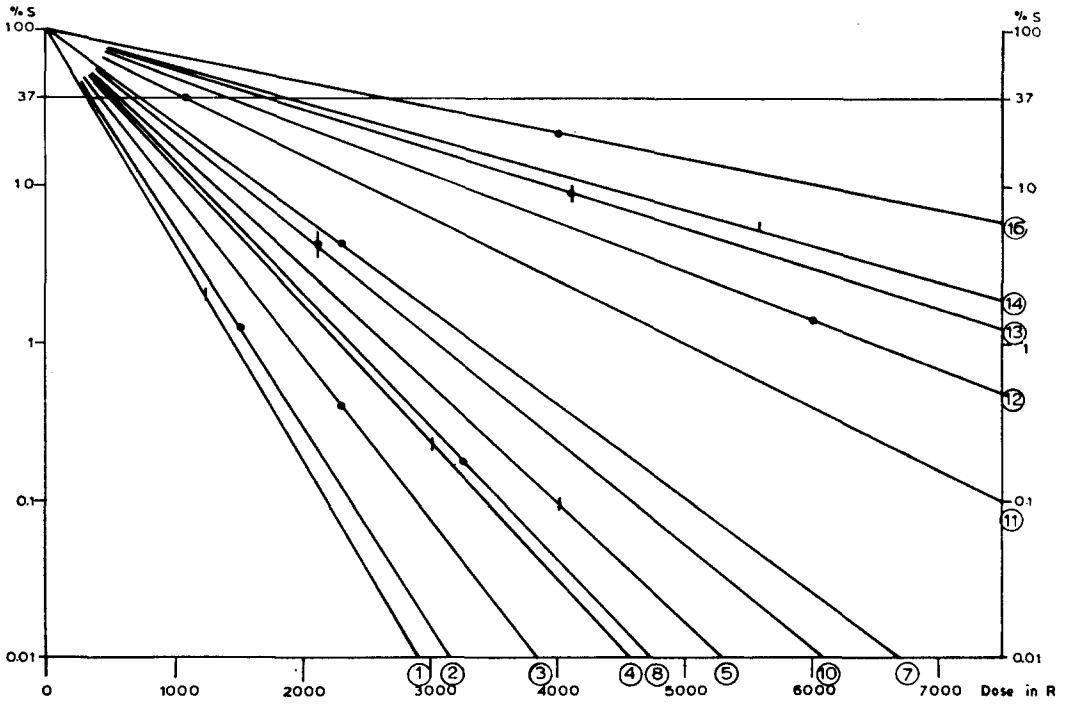

Fig. 9. *"Survival" percentages in thirteen patients in which pulmonary metastases received one series of irradiation.*

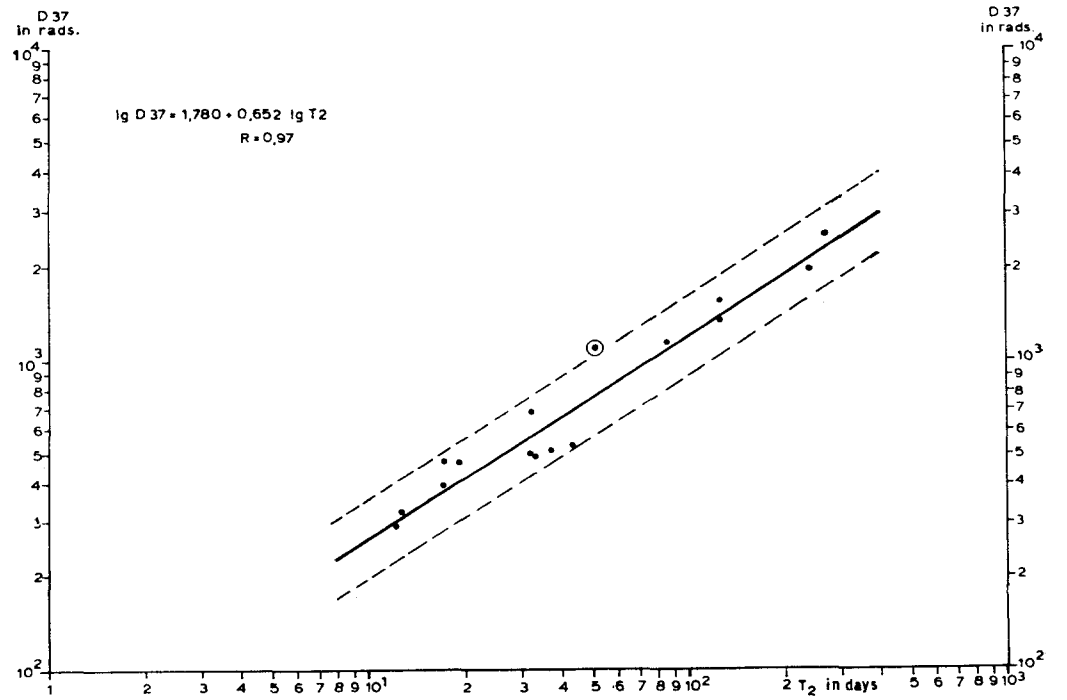

Fig. 10. *Relation between the logarithms of* D_{37} *and growth rate* (T_2).

short doubling time and, on the other hand, high values in cases with a long T_2. In Fig. 10, the relationship is visualised by plotting logarithms of D_{37} against the logarithms of T_2 according to the formula
$\log D_{37} = \alpha + \beta \log T_2$. Analysis by the Mathematical Centre in Amsterdam showed a positive correlation between the variables, and the equation for the regression line: $\log D_{37} = 1{\cdot}780 + 0{\cdot}652 \log T_2$.

In this calculation, patient no. 11 had to be left out of the series because the values for T_2 and D_{37} might not have been reliable. Several metastases showed some central necrosis. Moreover, the rather small total dose of 1088 R resulted in a high value of survival (37 per cent), giving more chance of error. In the curve, the established value of D_{37} is situated just outside the limits of confidence.

Examining the possibility of a linear relationship according to the formula
$D_{37}\ r = a + b\ .\ T_2$ it can be seen in Fig. 11 that the D_{37} values for both tumours with a doubling time of more than 150 days remain too low. When one disregards these two cases, there is a linear relationship for the remaining cases, giving the formula:
$D_{37} = 218{\cdot}4 + 10{\cdot}1\ T_2$, or roughly,
$D_{37} = 220 + 10\ T_2$.

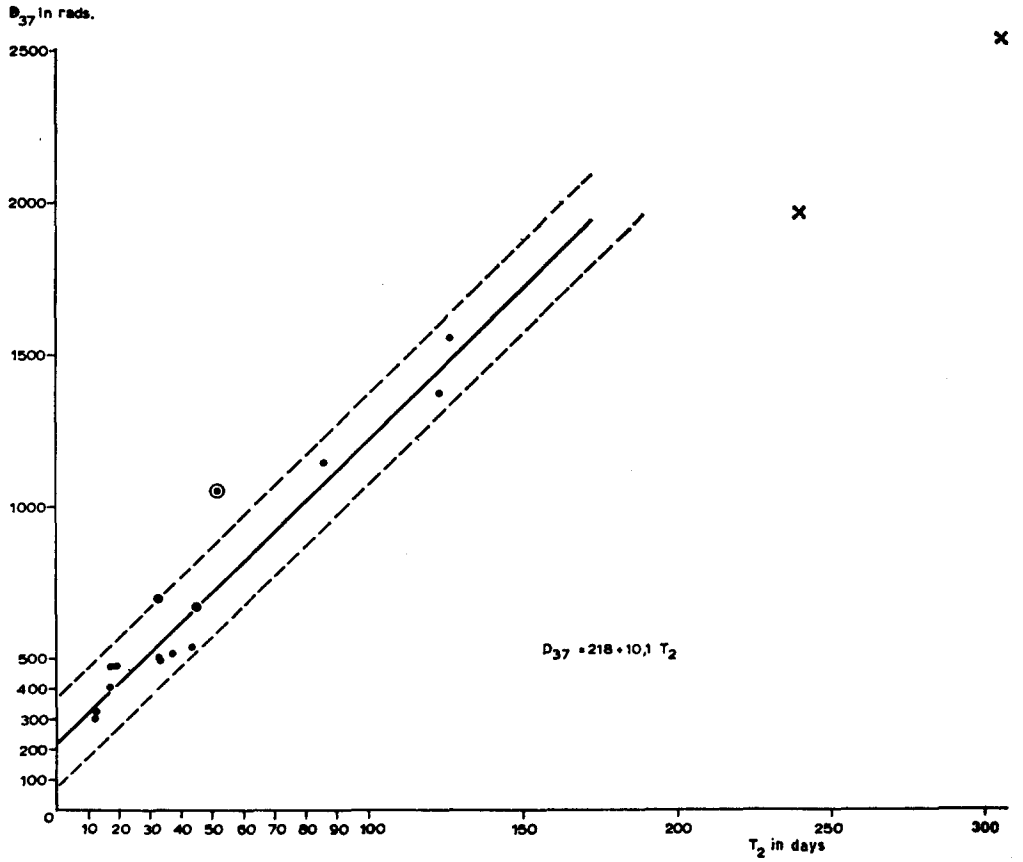

Fig. 11. *Relation between* D_{37} *and* T_2 *of pulmonary metastases for all patients examined with exception of the two cases with the most slowly growing tumours.*

DISCUSSION

The rather simple relationship found to exist between radiosensitivity and growth rate seems to confirm the old "law" of Bergonié and Tribondeau [30, 31], based on the rather small foundation of their experiments concerning the histological effect of radiation on spermatogenesis in rats. Also, it is in agreement with decades of clinical experience in radiotherapy. Most important, however, seems to be the possibility of describing the radiosensitivity of an individual tumour in a quantitative way, enabling one to estimate from the established growth rate the radiation response which can be expected. Several problems remain to be solved.

First of all, it should be noted that in the present study the measurements of growth rate and radiation effect were performed on pulmonary metastases, so that we have no proof that the conclusions also apply to primary tumours or other growths. However, at least until now, there are virtually no arguments available indicating that the basic principle presented here is not valid for all human tissues and tumours. In many respects, blood-borne pulmonary metastases offer one except-

Table 2. Experimental data for comparison of growth rate and D_{37}.

Authors		Object	T_2 (hr)	D_{37}*
Lockart, Elkind	(24)	Hela S_3 cell line 1	20·9	105
		cell line 2	17·8	97
		cell line 3	19·2	127
Puck, a.o.	(1)	Hela S_3	20	139†
Bases	(41)	Hela S_3	22±1	122
Elkind	(23)	Chinese hamster, ovary and lung	12	128
Sinclair	(45)	Chinese hamster, lung V 70–1	27	145
Barendsen, Reinhold	(42)	Rat: rhabdomyosarcoma cells	41	197‡
Barendsen, a.o.	(10)	Human kidney cells	20	135
Dewey, a.o.	(43)	Mouse L.P. 59 cells	24	145
Hewitt, Wilson	(6)	Leukaemia cells, mouse	13	157
Belli, Andrews	(44)	Lymph. Leukaemia	14·2	123
Suit, Shalek	(47)	Mouse, transpl. adenocarc. mammae	120	229–306§

*Values are presented in rads by approximation, using a factor 1R=0·96 rad for telecobalt as well as for orthovoltage therapy and a R.B.E. of 0·85 for cobalt 60 and 2·5 MeV radiation.
†Recalculation advised by Marcus with factor 1·45.
‡Personal communication.
§Value for T_2 is approximation derived from growth curves in the article. Values of D_{37} were calculated from those for irradiation under anoxic conditions by using factors 0·5–0·67 of the first, being O_2 factor given by the authors for the skin reaction.

tionally good possibilities for studying growth and radiation effects. Obviously, neoplastic tissue can grow undisturbed in the rather homogeneous, richly vascularized tissue of the lungs, where it is not hampered by denser structures as it would be in most other organs. This freedom of growth is reflected in the spherical shape, which also suggests an origin in an embolus of one or only a few cells. The sharp contrast assumed by its shadow against the air in the lungs on the roentgenological document allows the tumour to be observed during growth and regression without harming the patient or tampering with the biological process. In a way, these pulmonary metastases can be regarded as a culture *in vivo* of a pure cell line, and are comparable with cell cultures *in vitro*.

It is remarkable to observe how regression of the diameter of pulmonary metastases started very shortly after the beginning of a course of irradiation. It must be assumed that, perhaps owing to the rich vascularization, fatally damaged cells were quickly eliminated from the lungs. The cytological observations made on needle biopsies of radiosensitive tumours during irradiation by Johanson and Zajicek [32], suggest that in some instances rapid transport of dead cells occurs. From experience in radiotherapy it is well known that in most primary solid tumours and metastases outside the lungs, regression of volume first can usually be observed only several days or weeks after the beginning of irradiation, and that it often continues for several weeks after the last irradiation. Under these circumstances, lethally damaged cells may remain *in situ* for a longer period, disappearing only after lysis has taken place. It is possible that regenerative processes in the stroma and inflammatory infiltration retard regression of the measurable volume.

The resumption of exponential growth at the original rate shortly after irradiation is also remarkable. This observation is in agreement with data from cell culture experiments. It seems that the clinician's notion that radiation results in a diminished growth rate must be abandoned. It follows, that the development of a tumour is set back by irradiation, by a time interval equal to the time necessary to again reach the original volume. This means that a dose of radiation which gives but unsatisfactory reduction in the diameter of a slowly growing tumour still can effect a considerable prolongation of survival time. An example is shown in Fig. 5. Though each of two irradiation series caused only a slight reduction in the diameter of the metastases, it can be seen that it took about 4 years for the metastases to regain their original size, and that in this case, where no other metastases were present, life was prolonged by an equal period. Thus, apparently striking effects achieved with rapidly growing tumours can in fact prove to be less effective in terms of survival time.

The wide spread among the values for D_{37} encountered in this investigation seems to contradict the relatively small differences in D_{37} found in experiments with cell cultures and transplanted tumours. Most authors mention a D_{37} of between 100 and 200 rads for mammalian

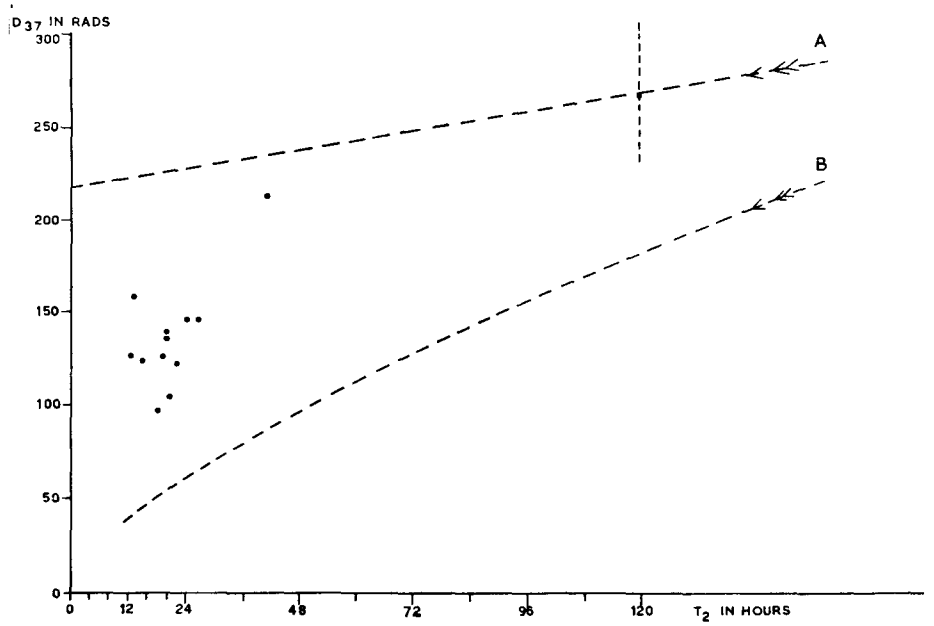

Fig. 12. *Values of* D_{37} *from experiments in relation to the concomitant doubling times of the cell lines. Curve A constitutes the extrapolation of the curve in Fig.* 11, *curve B of that in Fig.* 10.

cells under conditions of normal oxygenation, and an increase by a factor of 2 or 3 under anoxic conditions. This brings most radiobiologists to the conclusion that all mammalian cells have the same intrinsic radiosensitivity. It can be seen, however, that nearly all the investigated cell lines in these studies had a regeneration time of about one day. According to our formula $D_{37} = 220 + 10\ T_2$, small differences in T_2, e.g. from 0·5 up to 2 days, will have little influence on the value of D_{37}. On the other hand, it can be seen that conclusions gained from experiments with such rapidly growing tumour cell colonies cannot be extended to the effects of radiation on human tissues and tumours, most of which grow much more slowly. Probably, those tissues in man with a very high turn-over rate, such as those in the reproductive organs, bone-marrow and the epithelial lining of the small bowel can more closely be compared with the experimentally used cell lines [33–40]. In fact, it is these very tissues which are also known to be extremely radiosensitive.

Figure 12 shows values of D_{37}, obtained in experiments, plotted against the doubling times that were indicated by the various authors [1, 6, 10, 23, 24, 41–45]. As can be seen, most of the points are situated rather close together for cell lines with a doubling time of 0·5–1 day. The value for D_{37} of about 370 rads is deduced from the article of Suit and Shalek [46, 47]: the growth curves which they presented suggested a doubling time for the transplanted adenocarcinoma of about 5 days. Barendsen isolated a clone of rhabdomyosarcoma cells with a relatively low growth rate ($T_2 = 41$ hr), and found a D_{37} of 197 rads for these cells. Thus, there is an indication that in experiments, also, a tendency exists for more slowly growing colonies to be less radiosensitive.

It must be borne in mind that the formula given for D_{37} in relation to doubling time is only valid for fractionated irradiation with daily tumour doses of 100–150 rads. According to the curves shown by Elkind, a lower daily dose will result in a higher value of D_{37}. This may explain the effect of fractionating in radiotherapy, illustrated by the Strandquist curve. According to our formula $\log D_{37} = 1{\cdot}780 + 0{\cdot}652\ T_2$ the 37 per cent survival dose after fractionated irradiation would be about 60 rads for a tumour with a doubling time of 1 day. Using the simpler formula $D_{37} = 220 + 10 \cdot T_2$ would indicate a D_{37} of 230 rads for a T_2 of 1 day. This latter value seems more consistent with the expectation of a higher value for fractionated irradiation than the published values for single dose irradiation as practised in experimental work.

It certainly is not obvious that vascularization and oxygen tension are less pronounced in radio resistant tissues and tumours than in radio sensitive ones. Necrosis probably is seen more often in rapidly growing, radio sensitive tumours, than in slowly growing, radio resistant growths, but even allowing a sensitivity factor of three between normal and completely

anoxic conditions, this could not account for the differences in sensitivity encountered in our series, which correspond to a factor of more than eight. For the explanation of the influence of growth rate on radiosensitivity one could point to the differences in sensitivity known to occur during the cell cycle. One can refer to investigations with synchronously dividing cell colonies [48–53]. In general, the greatest radiosensitivity is found during the phases of the cycle prior to mitosis (S and G_2 phases) and the smallest during the interphase (G_1 phase). The differences established experimentally are probably not sufficient to explain the phenomena observed in our study, but again it must be emphasized that these experiments were done on cell lines with very short generation times. It would be interesting to know the sensitivity of cells with a long generation time, during their long period of rest, in comparison with that in the period during and directly preceding mitosis.

In our study, growth and regression were measured on tumours consisting of both tumour cells and stromal elements. In view of the constant growth rate found, one can assume that new, vascularized stroma is formed around existing structures and vessels at a rate comparable with that at which the tumour grows, and that this stroma constitutes a rather constant fraction of the total volume [54].

The most important consequence of the established correlation could be the possibility of estimating the total dose necessary to eradicate an individual tumour if its volume and growth rate are known. Investigating the growth rate of a primary tumour, it must be seen whether mitosis frequency counts in biopsy specimens, or techniques with labelled thymidine can be of help [55, 56]. The value D_{37}, indicating radiosensitivity, is based on statistical considerations in the target theory ($^{1}_{e}$). For clinical purposes, a value of D_{10} (equal to $2{\cdot}30 \times D_{37}$), i.e. the dose which would reduce the tumour volume to 10 per cent of the original, would be more practical, as it would make it simple to calculate the dose required to give a 90 per cent chance of killing the last tumour cell. If for example a tumour of 2 cm dia., comprising about 10^9 tumour cells, should have a doubling time of 18 days, a D_{37} of 400 rads (and a D_{10} of 920 rads) could be assumed if a mean daily dose of about 100 rads was given. A dose equal to $9.D_{10}$ should then leave about one cell capable of unlimited growth, and a dose of $10.D_{10}$ would offer a 90 per cent chance of preventing any cell from further regeneration. One would thus arrive at a dose of 9200 rads, applied over the course of about 3 months in this instance. Of course, in practice, a much higher daily dose would be used for treating a small primary tumour than was used in the palliative treatment of pulmonary metastases in our series, so that the total dose and treatment time would be considerably less. Of course different factors would have to be established for irradiation with daily doses differing from those of 75–150 rads, which were applied in our cases. The construction of survival curves for single dose irradiations would be of great help. An attempt in this direction will be undertaken.

Incidentally, it can be mentioned that the methods described in this study can also be used to obtain more exact data regarding the effects of chemotherapy [57, 58] on human tumours. It is hoped that the results which we have shown will stimulate clinical research on the radiosensitivity of malignant tumours, leading from a purely empirical approach to a more scientific basis for radiotherapy.

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RÉSUMÉ

L'influence des radiations ionisantes sur les métastases pulmonaires peut être clairement mise en évidence par la mensuration des lésions sur des radiographies successives. Une irradiation fractionnée provoque une réduction concentrique de la taille des métastases. Lorsque la mesure du diamètre est suffisamment précise, il est possible de calculer le pourcentage de réduction de leur volume. De telles études quantitatives ont été faites chez 16 *malades. La réduction de volume est déjà évidente quelques jours après l'administration de la première dose. La répétition d'une dose quotidienne constante permet d'établir une courbe exponentielle de survie. La métastase reprend sa croissance immédiatement après*

l'arrêt de la série d'irradiations, ou, au plus tard, quelques jours après celui-ci, et avec la même vitesse qu'avant le traitement. Les faits observés sont en accord avec les expériences d'Elkind sur l'irradiation fractionnée des cultures de cellules. Ainsi, la dose létale D_{37}, *définie comme la dose laissant* 37% *du volume initial de la tumeur capable de reprendre la croissance, peut être calculée chez des patients irradiés par les rayons* X *à* 250 *kV ou par le Cobalt*^{60}. *Les résultats ont montré de grandes différences de radiosensibilité parmi les tumeurs de différents types. Les valeurs de* D_{37} *allaient de* 300 *à* 2550 *rad. Ces différences ne peuvent être expliquées ni par l'hypothèse que toutes les cellules ont la même radiosensibilité intrinsèque, ni par des différences de la tension d'oxygène dans les tissus.*

On observa une bonne corrélation entre la radiosensibilité (D_{37}) *et la vitesse de croissance d'une tumeur, exprimée par son temps de doublement* (T_2). *Pour tous les cas, l'équation log* $D_{37} = 1{,}780 + 0{,}652 \log T_2$ *correspond aux données observées, dans des limites acceptables. En excluant les valeurs de* D_{37} *pour les deux tumeurs aux croissances les plus lentes* ($T_2 > 150$ *jours*), *toutes les valeurs de* D_{37} *se répartissent sur une droite correspondant à l'équation* $D_{37} = 220 + 10T_2$.

La signification de ces relations est discutée du point de vue de la radiobiologie clinique, et du point de vue de la pratique de la radiothérapie. La possibilité d'estimer la dose létale pour une tumeur, si l'on connaît son volume et sa vitesse de croissance, est d'un intérêt particulier. De nombreuses recherches supplémentaires, dans divers domaines, restent cependant à faire pour atteindre ce but.

SUMMARY

Pulmonary metastases from different types of tumour in Man show constant growth rates when measured accurately on successive roentgenograms of the chest. The influence of ionizing radiation on these metastatic tumours could be shown very clearly. Fractionated irradiation resulted in a concentric reduction in the size of the metastases, the outlines of which remained remarkably well defined. This made it possible to calculate the percentage of reduction in volume by measuring the change in diameter. Data on such pulmonary metastases in sixteen of our patients were suitable for quantitative analysis. It was found that volume reduction became evident a few days after the first dose was administered. Repetition of the same daily dose caused percentages of reduction, giving an exponential survival curve. After the last dose of a series was given, the tumour resumed in growth immediately, or at least within a few days, at approximately the same rate as before irradiation. The surviving fractions found in these cases did not conflict with the assumption of an exponential survival curve, as constructed by Elkind for fractionated irraditions of cell cultures. In this way, the mean lethal dose D_{37} *defined as the dose leaving* 37 *per cent of the original volume capable of resuming growth, could be calculated for these irradiations, in which daily doses of* 100–150 *rads were applied using* 250 kV *roentgen and Cobalt-*60. *The results showed wide variations in radiosensitivity amongst tumours of different types* (*Table* 1). *Values of* D_{37} *extending from* 300 *up to* 2550 *rads were encountered. These differences, which correspond to a factor of* 8·5 *cannot be explained by the assumption that all cells have the same intrinsic radiosensitivity and that all differences observed are caused by differences in oxygen tension in tissues.*

Good correlation was found to exist between the radiosensitivity (D_{37}) *and the growth rate of a tumour, expressed in terms of the doubling time,* T_2. *For all cases, the equation log* $D_{37} = 1{\cdot}780 + 0{\cdot}652 \log T_2$ *fits the data within fair limits. Excluding the value of* D_{37} *for two tumours, i.e. those with the lowest growth rate* ($T_2 > 150$ *days*), *all values of* D_{37} *fitted well in a linear correlation according to the equation,* $D_{37} = 220 + 10\ T_2$.

Possible explanations of the phenomena found are discussed, taking into account the concepts of modern experimental radiobiology. The significance of these relationships for clinical radiobiology and the practice of radiotherapy is considered. The possibility of estimating the lethal dose for a tumour if its volume and growth rate are known is of particular interest. Many additional investigations in various fields will be required to reach this goal, however.

ZUSAMMENFASSUNG

Lungenmetastasen ausgehend von verschiedenen menschlichen Tumoren, weisen eine konstante Wachstumsgeschwindigkeit auf, wenn man dieselbe genau auf Reihenröntgenbildern ausmisst. Der Einfluss von ionisierenden Strahlen auf die Metastasen kann klar bestimmt werden. Fraktionierte Bestrahlung führte zu einer konzentrischen Verkleinerung des Umfangs der Matastasen, deren Grenzen aber bemerkenswert klar definiert bleiben. Diese Feststellung ermöglicht es den Prozenentsatz der Volumenabnahme durch die Bestimmung des Durchmessers der Matastasen zu errechnen. Der Autor verfügt über genügende Einzelheiten über 16 *Patienten, um eine quantitative Analyse zu ermöglichen. Er fand dass wenige Tage nach der ersten Strahlendosis eine Volumenabnahme bemerkt wird. Die Wiederholung derselben täglichen Dosis erzeugte einen Prozentsatz der Abnahme, welche zu einer exponentiellen Ueberlebenskurve führte. Sobald die letzte Dosis einer Serie verabfolgt worden war, begann sofort oder innerhalb weniger Tage ein neues Tumorenwachstum und zwar mit der selben Geschwindigkeit wie vor der Behandlung. Die Ueberlebenszeiten stimmen mit der von Elkind ausgearbeiteten exponentiellen Kurve für fraktionierte Bestrahlungen von Gewebskulturen überein. Auf diese Weise konnte die mittlere letale Dosis* D_{37}, *welche als die Dosis, die* 37 *Prozent des ursprünglichen Volumens mit erhaltener Wachstumsfähigkeit überleben lässt, definiert wird, errechnet werden unter Verabreichung von* 100–150 *rad pro Tag durch Anwendung entweder von* 250 *kV-Röntgenstrahlen oder von Kobalt-*60. *Die Resultate ergaben grosse Unterschiede der Strahlenemfindlichkeit der verschiedenen Tumortypen. Werte für die* D_{37} *von* 300 *bis zu* 2550 *rad wurden gefunden. Diese Unterschiede, die einem Faktor von* 8,5 *entsprechen, können nicht durch die Annahme, dass alle Zellen dieselbe Strahlenempfindlichkeit haben, erklärt werden und ebensowenig durch die Hypothese, dass alle Unterschiede auf Differenzen in der Sauerstoffspannung innerhalb der Gewebe beruhen.*

Gute Uebereinstimmungen wurden gefunden, zwischen der Strahlenemfindlichkeit (D_{37}) *und der Wachstumsgeschwindigkeit gemessen an der Verdopplungszeit* (T_2). *Für alle Fälle entspsircht die Gleichung log* $D_{37} = 1{,}780 + 0{,}652$ *log* T_2 *ziemlich genau den Beohachtungen. Wenn man zwei Tumofen mit langsamer Wachstumsgeschwindigkeit* ($T_2 > 150$ Tage) *aus der Aufstellung der* D_{37} *fallen lässt, stimmen alle Werte für* D_{37} *mit der lineären Korrelation laut der Gleichung* $D_{37} = 220 + 10\ T_2$ *überein.*

Der Verfasser bespricht die möglichen Erklärungen seiner Befunde im Lichte der modernen experimentellen Radiobiologie. Die Wichtigkeit derselben für die klinische Radiobiologie und für die radiotherapeutische Praxis wird ebenfalls erwähnt. Die Möglichkeit, die letale Dosis für einen Tumor, wenn dessen Volumen und Wachstumsgeschwindigkeit bekannt sind, zu errechnen, erscheint ihm von besonderem Interesse zu sein. Selbstverständlich sind noch weitere Forschungen auf verschiedenen Gebieten notwendig, um dieses Ziel zu erreichen.

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Cytogenetical and Proliferative Characteristics of Acute Promyelocytic Leukaemia Cells*

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FOLLOWING the original description of acute promyelocytic leukaemia ("leucose aiguë à promyélocytes") by Croizat and Favre-Gilly [1], several cases have been successively reported [2–6]. The feature which distinguished this form of acute leukaemia from the other cytological varieties is the complete prevalence of a single cell type with the characteristics of the neutrophil promyelocyte.

The main haematological characteristic of this form is the presence of a maturation defect at a more advanced stage than in the commonest forms of acute leukaemia. Nevertheless, its clinical course is basically the same as that of acute leukaemias. If anything, it can be distinguished clinically by the fact that its course is more dramatic and there is a marked tendency to haemorrhage which has been shown to be due to an increased fibrinolysis, peculiar to these forms [5,6].

Some recent investigations on the proliferation and maturation capacity of acute leukaemia cells have shown that maturation is a basic defect in these cells and that this is responsible in its turn for reduced proliferative activity in the blast cell population as a whole [7].

Another interesting feature of acute leukaemia cells discovered in the last few years is the existence of various types of inconstant chromosome abnormalities in many cases [8–13].

In this paper are reported some kinetic and karyological investigations performed on one case of acute promyelocytic leukaemia.

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CLINICAL DESCRIPTION

O.B., aged 40, a worker with no history of occupational diseases. In the patient's history only recurrent episodes of bronchial asthma occurred.

About 1 month prior to being admitted, various haemorrhagical manifestations had appeared, expecially gum haemorrhages and epistaxis, followed by a slight persistent fever, marked asthenia, widespread pallor and intense haematuria.

On admission, the patient appeared well-nourished and presented pallor of the skin and mucosas. Gum haemorrhages and expistaxis were present. The liver was palpable 4 cm below the costal margin and was painful. The spleen and lymphoglandular apparatus were macroscopically uninjured.

The red-cell count was 3,780,000 per mm^3 with haemoglobin 77%. The number of nucleated cells in the peripheral blood was 5000 per mm^3 represented 94% by promyelocytic cells, 3% by polychromatic erythroblasts, 2% by orthochromatic erythroblasts and 1% by neutrophil granulocytes. In the peripheral blood there were practically no platelets. The blood group was I O Rh^+.

The bone marrow showed marked monomorphism with promyelocytic type cells, characterized by a very fine reticulum and a variable number of nucleoli. Cytoplasm was abundant and there were numerous azurophil granulations and a certain number of specific neutrophil granulations. The peroxidase test on these cells was highly positive. Bone marrow cells were represented by 92% promyelocytic cells, 0·5% basophil erythroblasts, 3% poly-

chromatic erythroblasts, 4% orthochromatic erythroblasts and 0·5% neutrophil granulocytes.

Within a few days the patient's general condition deteriorated considerably: nucleated cells in the circulating blood rose to 15,400 per mm^3, with 95% of promyelocytes. There was also a sharp increase in temperature to above 40°C subcontinuous and an accentuation of haemorrhagical phenomena.

In spite of massive transfusional and steroid therapy, rapid obnubilation took place with exitus 5 days after admittance.

METHODS

For our karyotype studies, direct bone marrow examination was done. One millilitre of marrow was suspended in 5 ml of isotonic liquid containing colchicine 1: 500,000 and, after 1 hr at room temperature, osmotic shock and fixation were carried out. The slides were then stained with Giemsa solution.

For the study of the proliferative capacity of the promyelocytic cells, H_3–thymidine was employed as a DNA precursor (specific activity 14·8 c/mM): bone marrow sample was incubated with a rotating system at 37°C for 3 hr with 1 μc/ml of H_3–thymidine and then smeared. After fixation in Carnoy, autoradiographical preparations were performed with the dipping method employing Ilford L4 nuclear emulsion. After about 10 days' exposure the slides were developed, fixed and stained with *May–Grünwald–Giemsa.*

RESULTS

The karyotypes of 44 mitotic figures were examined. No morphological abnormalities were observed and, while the modal number of chromosomes was normal, a fair number of hypodiploid cells with 44 chromosomes (haplo 16 and haplo 19–20) was observed (Table 1 and Fig. 1).

Autoradiographical studies showed H_3–thymidine incorporation in 6·5% of the promyelocytes (much lower therefore than that of normal bone marrow blast cells and promyelocytes).

A marked heterogeneity of the percent of labelling was also observed within the leukaemia cell population. In Fig. 2 the percentage values of thymidine labelling are closely correlated to the sizes of the cells: the highest labelling, similar to that of normal bone marrow promyelocytes, was observed in the largest cells (17 μ average diameter).

Table 1

Number of chromosomes	44	45	46	47	Total
Number of cells	25	4	53	1	83

On the other hand, no significant differences were observed in the number of grains per labelled cell among the different cell classes.

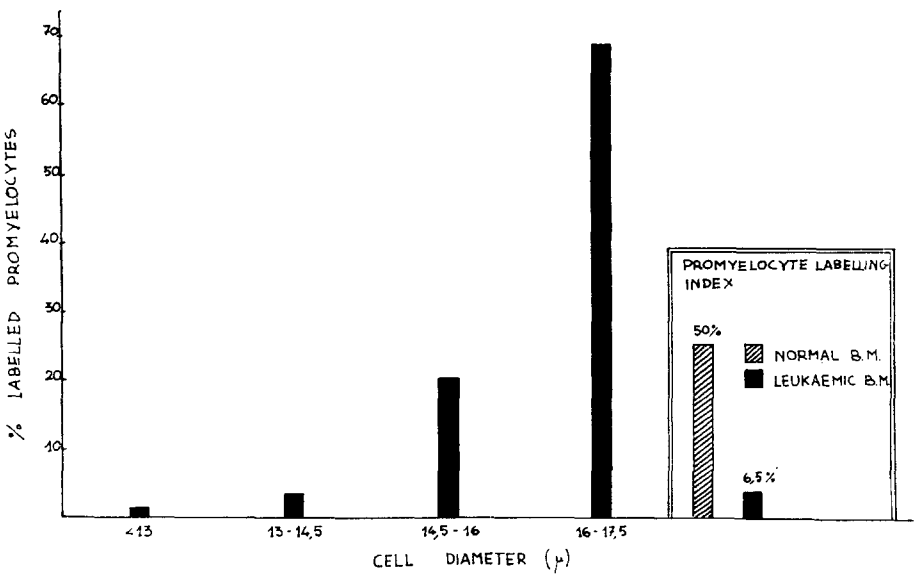

Fig. 2. Labelling index in the different promyelocyte cell classes.

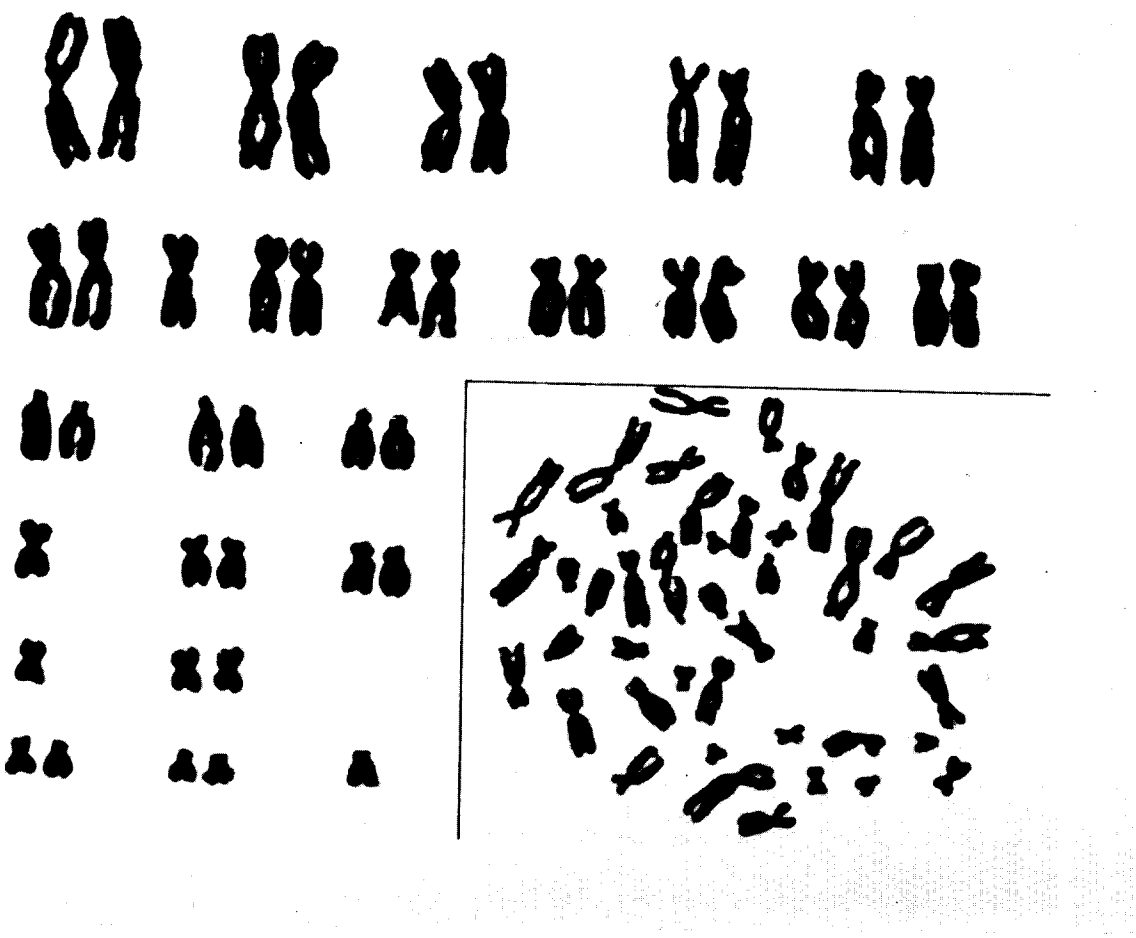

Fig. 1. *Karyotype from an hypodiploid cell* (*haplo* 16 *and haplo* 19–20).

(to face p. 190)

DISCUSSION

In acute leukaemia cells no specific karyotype abnormalities have been observed and a fair percentage of cases even showed a normal karyotype [8, 9, 12, 13]. On the other hand there is a constant karyotype abnormality in chronic granulocytic leukaemia cells (chromosome Ph^1) [10, 11]. In the present case of acute promyelocytic leukaemia, although characterized in the blood picture by partial neutrophil myeloid differentiation, the karyotype report (Ph^-) excluded any similarity between this form and chronic myeloid leukaemia. If anything, it was more like the reports commonly observed in acute leukaemias. In fact, in acute myeloblastic leukaemias in adults, a clear hypodiploid tendency has been reported [12].

It is well known that the proliferative capacity of acute leukaemia cells is lower as compared to that of normal bone marrow cells [14–16]. However in the same acute leukaemia cell population the proliferative capacity is heterogeneous, incorporation percentages being correlated to the cells diameters [17].

Both the low percentage of thymidine-labelled cells and the different proliferative activities of the population among the different cell classes indicate a similar behaviour between the present case of acute promyelocytic leukaemia and the other acute forms.

In acute leukaemias a certain aliquot of the cell population is endowed with a normal proliferative activity, which gradually decreases with the progressive ageing of the cells, incapable of maturation [7].

Since also in this case of acute promyelocytic leukaemia a proportion of the cell population shows normal proliferative capacities, one may consider the proliferative defect not as a primary fault, but as a consequence of the lack of differentiation.

As in the present case one may observe an attempt to differentiate towards the myeloid series, it seems that the decreased proliferative activity is unrelated to the stage at which such a defect is established.

This type of promyelocytic leukaemia must be clearly distinguished from chronic granulocytic leukaemias, in which normal maturation potentialities are associated with normal proliferative activities.

In conclusion, in this case both the cytogenetical investigation and the evaluation of the proliferative capacity showed features quite similar to those already observed in the blast cells of acute leukaemias.

Consequently, acute promyelocytic leukaemia can be classified under the heading of acute leukaemia not only for the clinical course of the disease and for the lack of differentiation of young myeloid cells but also for its cytogenetical and proliferative characteristics.

RÉSUMÉ

Dans un cas de leucémie aiguë à promyélocytes, nous avons réalisé une étude chromosomique, et nous avons évalué le pouvoir proliférant par l'incorporation de thymidine–H_3.

L'analyse du caryotype a montré un nombre modal de 46 *chromosomes et la présence de cellules hypoploïdes à* 44 *chromosomes* (30%).

L'étude de l'activité proliférative a montré des valeurs d'incorporation beaucoup plus faibles que pour les promyélocytes de moelle osseuse normale. L'ensemble de la population promyélocytaire a aussi montré des caractéristiques semblables à celles des leucémies aiguës en général.

Les études caryotypique et métabolique amènent à considérer la leucémie aigue à promyélocytes, bien que ses éléments soient partiellement différenciés, comme tout à fait semblable aux autres leucémies aiguës.

SUMMARY

A chromosome study was carried out in a case of acute promyelocytic leukaemia and the proliferative capacity was evaluated by means of H_3–thymidine incorporation.

Karyotype analysis showed a modal number of 46 *chromosomes with a fair percentage of* 44 *chromosome hypodiploid cells.*

Study of the proliferative activity which was carried out with H_3–thymidine gave clearly lower values in comparison with those of normal bone marrow promyelocytes. Further kinetic investigations within the promyelocytic populations also showed features similar to those reported in the other acute leukaemia forms.

Both karyotype and metabolic investigations suggest that acute promyelocytic leukaemia, in spite of partial differentiation, is quite similar to the other acute myelosis.

ZUSAMMENFASSUNG

Bei einem Fall von akuter Promyelozytenleukämie haben die Autoren die Chromosomen untersucht und ausserdum die Wucherungsfähigkeit mittels Einverleibung von Thymidin–H_3 beobachtet.

Die Analysen des Karyotyps ergaben eine Modalzahl von 46 *Chromosomen mit einer gewissen Anzahl von* 44 *Chromosomen enthaltenden hypodiploiden Zellen* (30%).

Die Untersuchung der Zellwucherungsfähigkeit mittels Thymidin–H_3 zeigte, dass diese deutlich geringer als die normaler, aus dem Knockenmark stammender, Promyelozyten ist. Andere Untersuchungen über die Kinetik dieser Promyelozyten ergaben ebenfalls Eigenschaften, die denen anderer leukämischer Zellen ähnlich sind.

Sowohl die Beobachtungen betreffend den Karyotyp als auch die betreffend den Zellstoffwechsel müssen zu dem Schluss führen, dass die akute Promyelozytenleukämie, trotz einer teilweisen Zelldifferenzierung, den anderen akuten Leukämieen ziemlich ähnlich ist.

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Carcinoma of the Bladder in Women

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INTRODUCTION

IT IS A wellknown fact that carcinoma of the bladder is more frequent among men than among women [1, 2].

The average age of patients with cancer of the bladder tends to be higher in women than in men, even if greater longevity of females in the general population is taken into account [2].

At the Rotterdamsch Radio-Therapeutisch Instituut (R.R.T.I.) we noted that a considerable number of female patients suffering from bladder cancer were unmarried. It also seemed to us that, in comparison with married female patients, spinsters were usually younger at the time of first treatment and the period elapsed after menopause was shorter.

Struck by the idea of a possible relationship between marital state, age, hormonal cycle and the effect of carcinogens on the bladder mucosa, the following statistical investigations have been carried out. Statistical calculations were performed by J. G. A. H. Kaalen, head of the statistical department of the R.R.T.I.

MATERIALS AND METHODS

Sex ratio was established for all patients with carcinoma of the bladder, treated at the R.R.T.I. between 1950 and 1963.

Sex ratio was also determined for groups according to tumour stage and to method of treatment in order to assess the significance of this ratio irrespective of the extensiveness of the growth.

The average age of male and female patients was calculated for the whole series and — in order to avoid a possible error due to selection — for each stage and treatment group.

Of female patients with complete records the marital state was compared with the Rotterdam population of the same age group, i.e. born between 1880 and 1915. Data concerning the Rotterdam population were provided by the Centraal Bureau voor de Statistiek in The Hague.

The interval between the onset of menopause and the first treatment of bladder cancer was calculated for unmarried and married patients. For the latter, the number of children was taken into account.

The possible relationship between the incidence of bladder cancer and the number of children in case of married women was investigated.

As data concerning the average menopausal age of Dutch women, born between 1880 and 1915 are not available, the average menopausal age of R.R.T.I. patients of the same age group (excluding breast cancer patients) was established for comparison.

RESULTS

In this series of patients with carcinoma of the bladder, men are more frequent than women, sex ratio being 3·2: 1.

This also holds good for patients grouped according to treatment [Röntgenirradiation (Rö), Telecobaltirradiation (Co^{60}) and Radiumimplantation (Ra)] and according to tumour stage [T_I = submucosal, T_{II} = muscular, T_{III} = perivesical, T_{IV} = pelvic fixation.

The average age of female patients is significantly higher than that of male patients. This same tendency can also be demonstrated in each stage and treatment group. (Figs. 1 and 2).

The proportion of spinsters is higher in female patients with bladder cancer (Ca) than in the female Rotterdam population (R) of the same age group. The same tendency persists in sub-groups according to age, except for age group 75–84 years (Fig. 3).

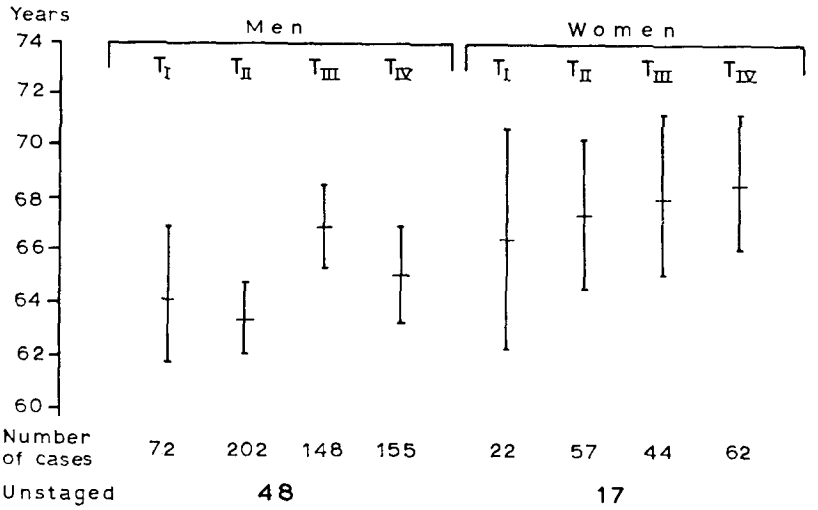

Fig. 1. Average age (with 95% conf. limits of average) of female and male patients with carcinoma of the bladder. According to tumour stage.

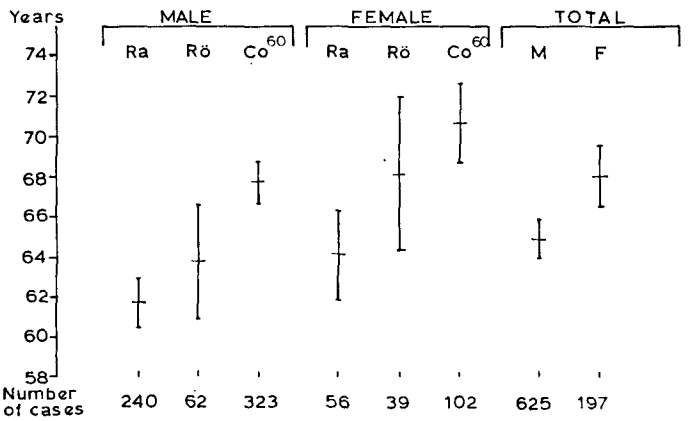

Fig. 2. Average age (with 95% conf. limits of average) of female and male patients with bladder cancer. According to method of treatment.

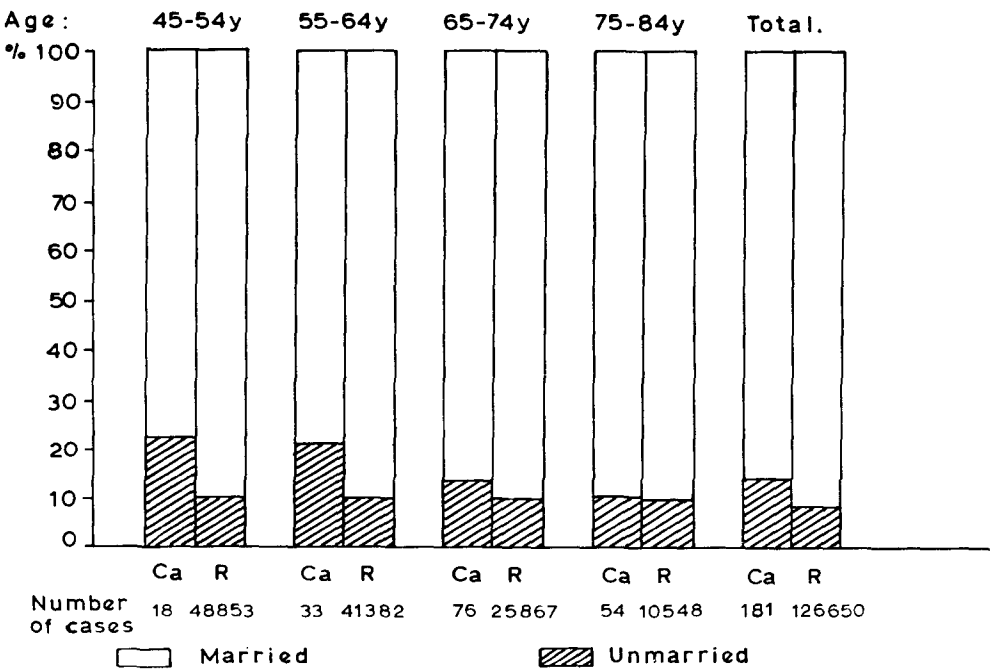

Fig. 3. Distribution of marital state among female patients with bladder cancer (Ca) and female Rotterdam population (R). According to age group.

The average age of unmarried female patients tends to be lower than that of married ones. The number of children of the latter shows no relationship with cancer age.

The mean interval between menopause and diagnosis of bladder cancer tends to be longer in case of married than in case of unmarried patients (Fig. 5). This is due to a higher average menopausal age (Fig. 6), and a lower average carcinoma age (Fig. 4) of spinsters. The number of children appears to have no bearing on this interval.

By comparing the average number of children of various female age groups it is shown that this average increases with age. This applies to both the Rotterdam population and patients with bladder cancer. The mean number of children of all age groups combined is significantly higher for women with bladder cancer than for the female Rotterdam population (Fig. 7).

The average menopausal age of bladder cancer patients is comparable to that of a control group, taken at random from the R.R.T.I.-material, excluding patients with breast cancer (Fig. 8).

From Fig. 8 one can see that the proportion of spinsters among the R.R.T.I. control women is similar to that of the Rotterdam population and different from the group of patients with bladder cancer.

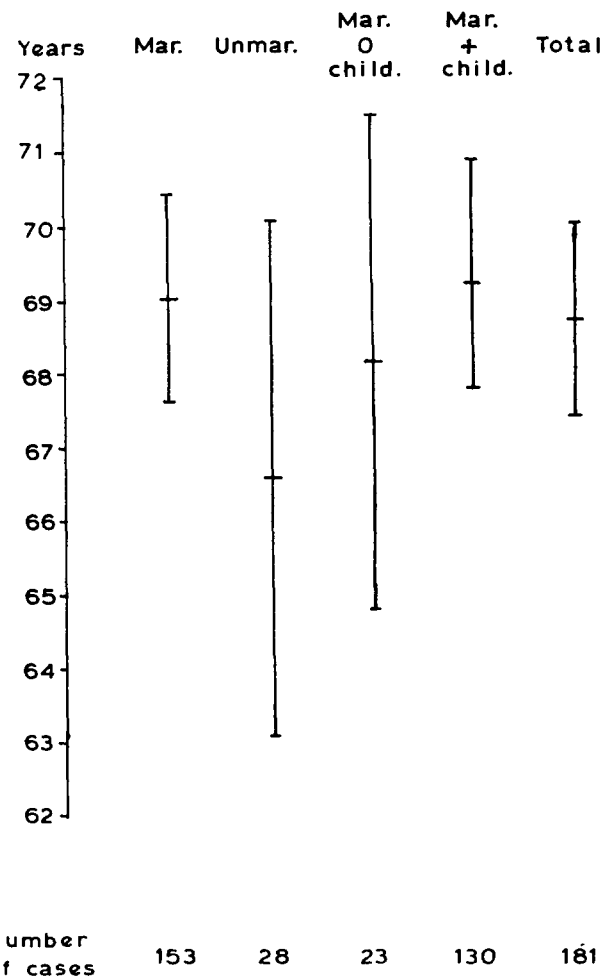

Fig. 4. Average age of female patients with carcinoma of the bladder (with 95% conf. limits of average). According to marital state and to having children.

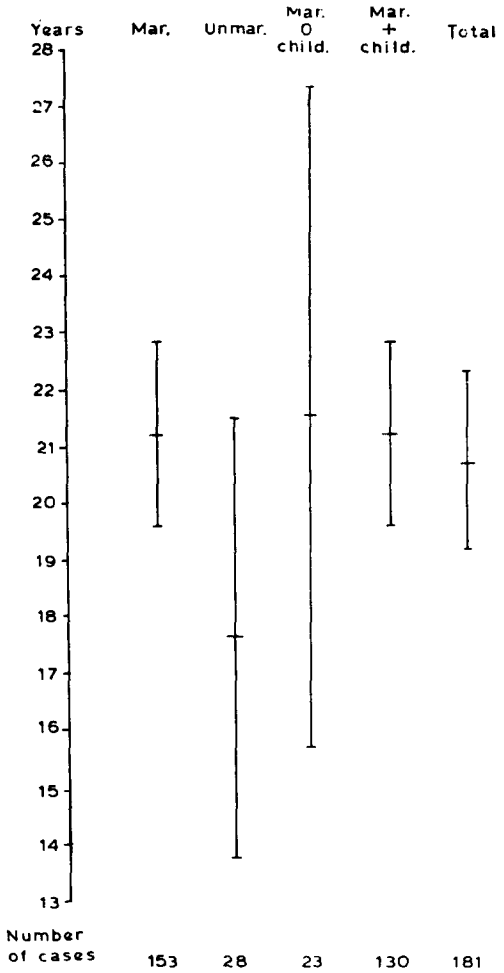

Fig. 5. Average interval between menopause and first treatment of female patients with carcinoma of the bladder (with 95% conf. limits of average). According to marital state and to having children.

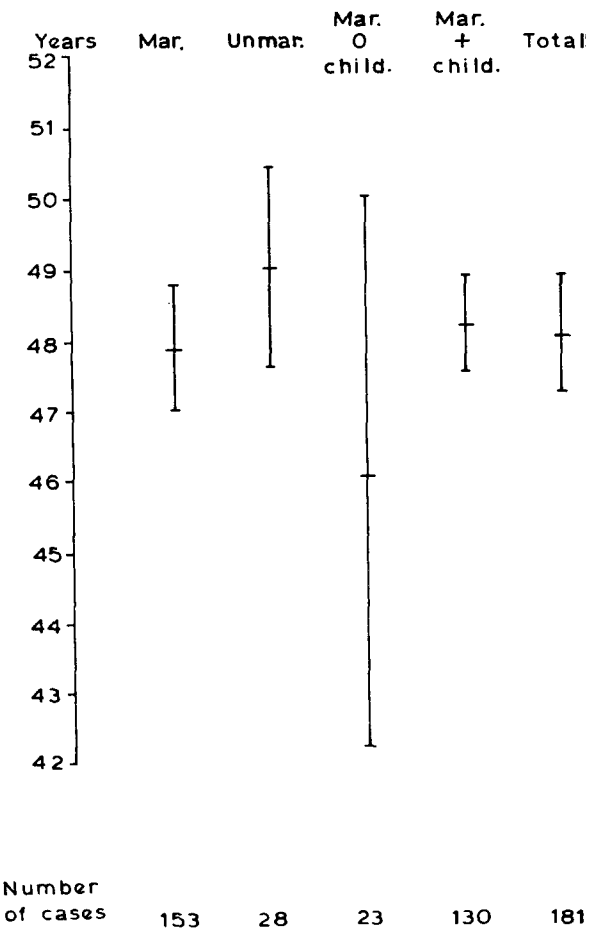

Fig. 6. Average age at menopause of female patients with carcinoma of the bladder (with 95% conf. limits of average). According to marital state and to having children.

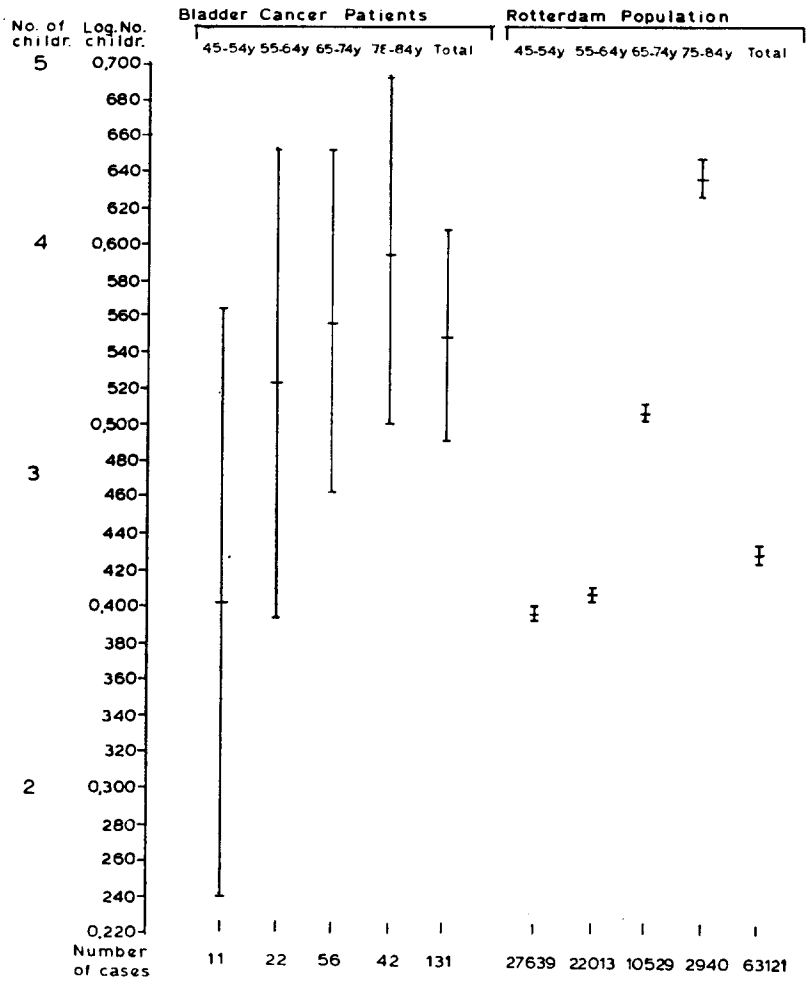

Fig. 7. Average number of children (with 95% conf. limits of average) of married women with children. According to age group.

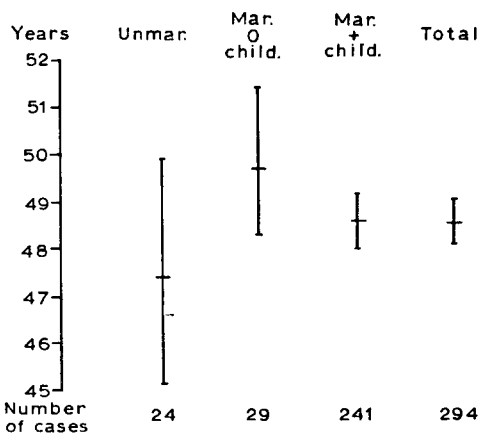

Fig. 8. Average menopausal age of cancer patients of R.R.T.I. (with 95% conf. limits of average). According to marital state and to having children. (Breast cancer and bladder cancer excluded).

DISCUSSION

Men are more likely to develop carcinoma of the bladder than women. Exogenic occupational carcinogens and smoking might account for this finding. Endocrinological differences may also be of importance.

In case of bladder cancer the average age of women is higher than that of men. Hormonal influence during reproductive age of women might act as either direct or indirect protection of the urothelium against carcinogens or as inhibitor of the carcinogens, thus both diminishing the carcinogenic effect and postponing the occurrence of the neoplasm in women. After menopausal age this hormonal effect might diminish.

The proportion of spinsters among our female patients with bladder cancer is higher than can be expected from the normal distribution in the Rotterdam population of the same age group.

Exposure to carcinogens is not likely to be different among married and unmarried women, as the latter had usually been doing domestic work, teaching or nursing. Smoking was equally unusual in both groups. Therefore one may assume that protective mechanisms are more pronounced in married than in unmarried females.

The average menopausal age in case of spinsters is higher than in case of married women. Assuming hormonal protection during reproductive age, one might expect bladder cancer at a higher age in spinsters than in married women. The reverse, however, is true. This again suggests an additional protection connected with marriage.

In the eldest age group (75-84 yr) the percentages of unmarried women among bladder cancer patients and among the Rotterdam population are equal. Hence it might be speculated that bladder cancer, diagnosed at this high age, originates from a period well after all protecting hormonal mechanisms have disappeared.

No reasonable hypothetical explanation has been found for the significantly higher number of children among married female patients as compared to married Rotterdam women of comparable age group.

Abnormalities in menopausal age of patients with bladder cancer, either married or unmarried, can be excluded, as it is comparable to the Rotterdamsch Radio-Therapeutisch Instituut control group and to figures concerning Western European countries as published by various authors [3, 4].

It might be worthwhile to check abovementioned hypotheses by animal experiments.

SUMMARY

Of 822 *patients with carcinoma of the bladder treated at the Rotterdamsch Radio-Therapeutisch Instituut between* 1950 *and* 1963 *the average age at first treatment was higher in females than in males.*

The proportion of spinsters among female patients with bladder cancer was significantly higher as compared with the Rotterdam female population belonging to the same age group. In spinsters the average interval between menopause and cancer was shorter than in married women.

The theory is put forward, that the female hormonal cycle might act protectively against the development of bladder cancer. This protection might be enhanced by some mechanism, related to marriage.

RÉSUMÉ

Sur 822 *malades traités au Rotterdamsch Radio-Therapeutisch Instituut, de* 1950 *à* 1963, *pour des cancers vésicaux, on a trouvé qu'au premier traitement, l'âge moyen des femmes était plus élevé que celui des hommes.*

Chez ces femmes malades, la proportion de célibataires surpassait, de façon statistiquement significative, celle des célibataires de mêmes âges dans l'ensemble de la population féminine de Rotterdam.

L'intervalle moyen entre la ménopause et l'apparition du cancer était plus court chez les femmes célibataires que chez les femmes mariées.

On peut donc proposer l'hypothèse que le cycle hormonal féminin forme une protection contre le développement du cancer vésical. Cette protection est probablement plus prononcée chez les femmes mariées.

ZUSAMMENFASSUNG

Bei 822 Blasenkrebspatienten des Rotterdamsch Radio-Therapeutisch Instituut übertraf das Durchschnittsalter der Frauen bedeutend das der Männer.

Der Prozentsatz unverheirateter Frauen war signifikant gröszer bei Blasenkrebskranken als bei der Rotterdammer Bevölkerung der gleichen Altersklasse.

Bei unverhairateten Frauen war die Periode zwischen Menopause und Krebsdiagnose kürzer als bei den Verheirateten.

Die Hypothese eines hormonalen Schutzes gegen Blasenkrebs bei Frauen wird erörtert. Dieser Schutz ist möglicherweise grösser bei verheirateten Frauen.

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Notes et Communications

Groupe Européen de Chimiothérapie Anticancéreuse (G.E.C.A.)

Protocole pour les Essais Cliniques de Traitement des Cancers Mammaires Humains en Phase Avancée

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CE PROTOCOLE, qui est inspiré du protocole établi par les "National Institutes of Health", est appliqué depuis plus de cinq ans par le "Groupe Coopérateur du Cancer Mammaire".

Une des activités essentielles du Groupe consiste dans l'évaluation des traitements par des composés nouveaux [1, 2]: hormones, leurs dérivés éventuellement dépourvus d'effet endocrinien, substances chimiques sans rapport apparent avec les précédentes. La rigueur nécessaire dans ce type d'étude ne s'obtient qu'en observant scrupuleusement un ensemble de règles garantes d'un jugement objectif; ces règles sont décrites ci-après. Deux études ont déjà été publiées: l'une compare l'effet de la delta-1-testololactone à celui du propionate de testostérone [3, 4]; l'autre compare l'effet de l'association delta-1-testololactone et propionate de testostérone à l'effet du propionate de testostérone seul [5].

Définition des essais cliniques

Deux types d'essais sont effectués; la méthode d'évaluation des résultats est identique. mais la méthode de sélection des malades est différente.

Seules les malades dont le cancer est trop avancé que pour être traité par la chirurgie ou la radiothérapie curatives font l'objet des études du Groupe. Les malades porteuses d'une tumeur mammaire autre qu'un carcinome, et celles présentant un autre cancer, en plus du cancer mammaire, sont exclues.

Les essais ne sont effectués qu'avec des substances présentant toutes les garanties quant à leur innocuité et sans jamais priver une malade d'un traitement reconnu, efficace.

Essai de type I

Dans ce type d'étude, on essaie de déterminer si une substance qui possède une activité anticancéreuse chez l'animal présente une certaine efficacité dans le cancer mammaire généralisé. Si, pour quinze malades traitées l'une après l'autre par ce composé, aucune rémission objective n'est observée, le produit est considéré comme inefficace; en effet, en procédant de cette façon, il y a 90 chances sur 100 pour que le produit testé ait une efficacité inférieure à 15%. Par contre, si une régression des lésions est observée parmi un nombre de malades égal ou inférieur à quinze, une étude de type no. 2 est entreprise.

Essai de type II

Il s'agit de déterminer dans quel pourcentage de malades la substance testée induit une rémission. Cette évaluation nécessite un

groupe témoin de malades auxquelles on administre une substance d'activité connue, considérée au moment donné comme le meilleur traitement médical du cancer du sein généralisé.

Description du protocole des essais du type II

Sélection des malades — La preuve histologique du carcinome mammaire doit être fournie.

— Les malades préménopausiques ou celles dont la ménopause naturelle date de moins d'un an sont exclues de l'étude [6]; cependant, si ces malades ont subi une ovariectomie bilatérale ou une castration radiologique par administration d'une dose de 1200 r, elles peuvent être traitées à condition qu'une période de trois mois au moins se soit écoulée depuis la castration sans qu'une rémission se soit manifestée.

— Les malades ne peuvent avoir reçu de traitement hormonal préalable pour leur cancer, ni avoir subi de surrénalectomie ou d'hypophysectomie; ces thérapeutiques pourraient, en effet, modifier la réponse au composé administré lors de l'essai clinique. Un traitement hormonal antérieur, terminé depuis au moins trois mois, et donné pour des raisons autres que le cancer mammaire — donc à des doses faibles — n'exclut pas la malade de l'étude, à condition que les hormones n'aient pas été administrées sous une forme à action prolongée.

— Le début du traitement est précédé d'une période d'observation, au cours de laquelle des signes objectifs de progression des métastases doivent être notés.

— En cas de doute, la nature métastatique d'une lésion doit être confirmée par biopsie, quand cela est faisable.

— Les lésions osseuses très douloureuses, ou pouvant être le siège d'une fracture, doivent être irradiées; l'évaluation du médicament doit alors être basée sur les métastases éloignées de la zone d'irradiation.

— Les malades ayant comme lésion unique une métastase cérébrale ou un épanchement pleural sont exclues de l'étude, vu la difficulté d'évaluation objective de ces cas.

Bilan des lésions et techniques de mesure — Les lésions cutanées, sous-cutanées, ganglionnaires et mammaires sont mesurées à l'aide d'un pied à coulisse; leur plus grand diamètre et le plus grand diamètre perpendiculaire au premier sont mesurés et notés. En cas de lésions très étendues, avec placards d'ulcérations, et de nodules nombreux, un décalque sur feuille de matière plastique transparente est pratiqué. Les lésions visibles sont de plus photographiées dans des conditions identiques.

— Les métastases pulmonaires et hilaires sont mesurées à l'aide du pied à coulisse.

— Les masses abdominales, le foie métastatique, leurs distances par rapport aux repères habituels sont mesurés. Les tests hépatiques, la laparoscopie et la biopsie hépatique peuvent aider à l'évaluation.

— Les radiographies du squelette complet, à l'exception des parties distales des membres, permettent d'évaluer les lésions osseuses; le critère objectif d'amélioration consiste dans le dépôt de calcium dans une lésion ostéolytique, sans progression de l'ostéolyse. Les lésions d'emblée ostéoplastiques sont d'évaluation plus difficile.

— L'évaluation objective des métastases du système nerveux central est très difficile, car l'amélioration de certains signes peut être due à des facteurs indépendants de la croissance de la tumeur, par exemple à des fluctuations de l'oedème cérébral.

— Cette liste d'examens n'est pas limitative; les examens sont faits lors de la première visite de la malade, puis répétés toutes les quatre à six semaines, à l'exception des biopsies.

Evaluation de la réponse au traitement — L'amélioration subjective n'est pas prise en considération pour évaluer la réponse au traitement. Comme pour la progression des lésions avant le début du traitement, seuls des critères objectifs sont utilisés; ils consistent dans les modifications de volume des masses tumorales, car les changements biochimiques et hématologiques ne peuvent être considérés comme des critères irréfutables d'une modification de la maladie.

— Il y a deux réponses possibles au traitement: l'échec et la rémission objective. Voici les critères définissant celle-ci:

(a) diminution, de façon mesurable, de toutes les masses tumorales;
(b) diminution de plus de 50% des lésions non osseuses, alors que les métastases osseuses restent inchangées;
(c) diminution de plus de 50% des lésions, quelle que soit leur localisation, les lésions restantes ne se modifiant pas.

La progression d'une seule métastase, même si toutes les autres diminuent de volume, entraîne le diagnostic d'échec.

— Lors des évaluations des radiographies, l'observateur ignorera l'ordre chronologique réel des clichés. L'évaluation finale des cas doit être confirmée par un observateur n'ayant pas participé à leur traitement.

Classification des malades — L'éventuelle différence de réponse d'après le temps écoulé depuis

la ménopause et d'après la localisation anatomique des métastases [7] conduit à répartir les malades en douze catégories; l'ensemble des malades est d'abord divisé en quatre classes d'après le nombre d'années écoulées depuis la ménopause: moins de 1 an, de 1 à 5 ans, de 6 à 10 ans et plus de 10 ans après la ménopause. Chaque classe est divisée en trois catégories, d'après la localisation des métastases: (a) les lésions locales, cutanées et ganglionnaires; (b) les lésions osseuses; (c) les lésions viscérales. En passant d'une catégorie à la suivante, le pronostic devient plus défavorable. Une malade qui possède des lésions dans plusieurs systèmes est toujours classée dans la catégorie dont le pronostic est le plus mauvais.

Choix du traitement. Le nombre de malades à traiter est déterminé au préalable, afin d'obtenir des résultats statistiquement significatifs. Le groupe expérimental et le groupe témoin comporteront le même nombre de malades. C'est le hasard qui déterminera à quel groupe chaque malade sera affectée. En effet, un tirage au sort, effectué de façon autonome pour chaque clinique participante, décide pour un nombre pair de malades de chacune des douze catégories définies plus haut, quel traitement sera donné à chaque malade, suivant son numéro d'admission dans l'étude.

La méthode du double insu est utilisée, sauf si le groupe témoin reçoit une substance différente par son aspect ou ses propriétés biologiques du composé testé [8].

Durée du traitement. Si aucune régression ou aucune aggravation ne se manifeste, le traitement est arrêté au bout de six semaines. Si, avant ce moment, des signes objectifs de progression sont notés, le traitement est arrêté. De même, si des effets secondaires du composé mettent la vie de la malade en danger ou occasionnent des souffrances, le traitement est arrêté; la malade est alors considérée comme un échec de l'essai. En cas de rémission, le traitement est évidemment poursuivi jusqu'à l'apparition d'une nouvelle évolution des lésions. Si la rémission dure plus de quatre mois, la fréquence des examens décrits plus haut peut être diminuée.

Remarque

Les personnes désireuses d'obtenir des précisions concernant le protocole d'étude peuvent s'adresser au Professeur Tagnon, Service de Médecine Interne et d'Investigation Clinique, Institut J. Bordet, 1, rue Héger-Bordet, à Bruxelles.

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Announcements

The European Journal of Cancer has begun its second year. The policy of the Journal has been and will continue to be to publish papers of high quality in the experimental field as well as in clinical investigation. Our opinion is that the Journal will fulfill its destination and purpose better if it can bring together laboratory workers and clinical investigators. Therefore experimental work in the basic sciences as well as scientifically conducted clinical investigation will continue to be published in the Journal.

Le Journal Européen de Cancérologie a commencé la seconde année de son existence. Notre programme a consisté, dès le début, à publier des travaux expérimentaux de laboratoire, ainsi que des études cliniques de bonne qualité. Nous pensons que le Journal répond à sa destination en réunissant, dans une même publication, les chercheurs de laboratoire et les investigateurs cliniques. C'est pourquoi le Journal poursuivra la réalisation de son programme en faisant voisiner, dans ses colonnes, les sciences de base avec la médecine clinique scientifique.

Letters to the Editor

Beginning with No. 3, the European Journal of Cancer will accept "letters to the Editor" for prompt publication. The length of these short communications should not exceed 750 words including title, authors, bibliography and acknowledgements. They should furnish sufficient data to support their conclusions and conform to the usual standards of the Journal.

Lettres à l'Editeur

A partir du n° 3, le Journal Européen de Cancérologie acceptera, en vue d'une publication rapide, de courtes notes appelées "lettres à l'Editeur". Leur longueur ne doit pas dépasser 750 mots, y compris le titre, les noms d'auteurs, la bibliographie et les autres mentions. Elles devront fournir les données expérimentales ou d'observation nécessaires pour étayer leurs conclusions et se conformer aux instructions applicables aux articles ordinaires.

Briefe an den Herausgeber

Beginned mit der 3. Folge wird die "Europäische Zeitschrift für Cancerologie" "Briefe an den Herausgeber" für baldige Veröffentlichung annehmen.

Der Umfang dieser kurzen Mitteilungen sollte 750 Worte nicht überschreiten, einschliesslich Ueberschrift, Autoren, Bibliographie und andere Annerkungen. Sie sollten genügend Unterlagen enthalten, um die Schlussfolgerungen zu untermauern und allen übrigen Anforderungen der Zeitschrift entsprechen.

Erratum

A. H. GORDON: Synthesis of plasma proteins by the perfused rat liver. Effects of protein free diet, 3′-MDAB, and dimethylnitrosamine, *Europ. J. Cancer* **2**, 19–31 (1966)

Page 21, Table 1, footnote 1
for "3·93 *m mol leucine*" read "3·93μ *mol leucine*".

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A Factor Toxic to Chick Embryos in Blood of Tumor Bearing Animals*

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INTRODUCTION

IT WAS observed previously in these laboratories that whole blood and blood fractions from humans, mice and hamsters with neoplastic disease produced a high rate of mortality of chick embryos (40–100 per cent) within 7 days when inoculated onto the chorioallantoic membrane. In contrast to these observations, similarly implanted blood from normal and non-neoplastic sources was relatively innocuous and caused only a low percentage (0–30 per cent) of deaths in chick embryos [1, 2].

These studies have been extended to include samples of heparinized whole blood and plasma from a variety of normal animals, from those bearing a spectrum of neoplasms, animals with variations in normal physiology, and those with experimentally produced abnormalities [3].

MATERIALS AND METHODS

Embryonated White Leghorn chicken eggs of the Thompson strain§ were incubated at 38°C and were 7-days-old when implanted. At least seven to ten eggs or more were used in testing each specimen. A 0·25 cm^2 opening was made in the egg shell. The air sack was punctured, which allowed the embryo to drop away from the shell membrane. The portion of the shell membrane within the shell window was removed, thus exposing the chorioallantoic membrane. Implants were made in eggs by inoculation of 0·1 ml of fluid; such as, whole blood, plasma, or other material to be tested on the chorioallantoic membrane in the area of a large blood vessel. The opening in the shell was then covered with transparent cellophane tape and sealed with paraffin. Observations were made daily and experiments were terminated after 7 days.

All specimens were subjected to appropriate bacteriological examination, and specimens which were found to be contaminated were eliminated from further consideration, since such contamination invariably resulted in death of the embryo. Because some fluctuation in mortality of untreated chick embryos exists, specimens from normal animals always were tested simultaneously with those from animals bearing neoplasms. All blood samples were coded and handled by the technician performing the experiment in blind fashion.

RESULTS

Chick embryos were relatively unaffected by blood and plasma from a variety of normal animals (Table 1). The percentage of embryo mortality fell well within the range of 0–30 per cent in the 7-day period; and, therefore, these

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§Fertilized White Leghorn eggs were purchased from Thompson's Fairweather Farm, Andover, Massachusetts.

Table 1. Low mortality of chick embryos inoculated with blood of normal animals

Animal	No. of animals	Inoculum	No. of embryos dead/No. of embryos implanted	Dead (%)
Mouse*	65	Whole blood	32/323	9·9
	20	Plasma	5/98	5·1
Rat (W/Fu)	2	Whole blood	2/30	6·7
	2	Plasma	2/28	7·2
Chicken (White Leghorn)	8	Whole blood	15/133	11·2
	4	Plasma	7/68	10·2
Guinea pig	3	Whole blood	5/65	7·6
	5	Plasma	4/56	7·1
Dog	3	Whole blood	3/29	10·3
	2	Plasma	3/30	10·0
Syrian hamster	9	Whole blood	13/162	8·0
	3	Plasma	3/40	7·5
Rabbit	2	Whole blood	2/20	10·0
	2	Plasma	2/19	9·5

*Whole blood and plasma from six strains of mice of both sexes were inoculated in eggs.

specimens were considered to be non-toxic. There did not seem to be any appreciable difference in toxicity in eggs between whole blood and plasma.

Blood from animals with tumors which grew progressively as localized lesions, but which failed to metastasize, was also non-toxic in embryonated chick eggs (Table 2). These animals included cortisone-conditioned Syrian hamsters bearing transplantable human tumors in cheek pouches or in thigh musculature, mice bearing spontaneous and induced tumors, and animals bearing homologously transplantable tumors.

In marked contrast to these results, however, blood from animals with leukemia or lymphosarcoma was lethal in eggs (Table 3). The embryo mortality ranged between 50 and 100

Table 2. Low mortality of chick embryos inoculated with blood of animals bearing localized tumors

Tumor	Host	No. of hosts assayed	No. of embryos dead/No. of embryos implanted	Dead (%)
Human				
Myxofibrosarcoma	Hamster	7	6/63	9·5
Fibrosarcoma	Hamster	3	6/38	15·7
Malignant melanoma	Hamster	3	3/38	7·8
Wilm's tumor	Hamster	3	8/38	28·5
Hamster				
Ascites sarcoma	Hamster	3	5/39	12·8
Sarcoma	Hamster	2	6/24	25·0
Mouse				
Adrenotropic pituitary tumor	LAF_1	15	9/73	12·3
Mammary adenocarcinoma	C3H	8	4/38	10·5
Mammary adenocarcinoma	C57BL/6	6	0/27	0·0
Pleomorphic cell sarcoma	C57BL/6	4	1/20	5·0
Sarcoma (induced)	Swiss	6	2/30	6·6
Spon. mammary tumor	C3H	10	4/47	8·5
Rat				
Mammo-somatotropic pituitary tumor	F/Fu	2	1/19	5·2

Table 3. *Lethal effect on chick embryos inoculated with blood from animals with leukemia or lymphosarcoma*

Disease	Host	No. of hosts assayed	No. of embryos dead/No. of embryos implanted	Dead (%)
Leukemia	Mouse strain			
P1534	DBA/2	3	15/15	100·0
L1210	DBA/2	5	20/24	83·3
AK4	AKR	32	97/158	61·3
AtT18VCLK	LAF_1	6	29/29	100·0
C1498	C57BL/6	6	25/28	89·3
Lymphosarcoma				
6C3HED	C3H	3	10/12	83·3
RL	Syrian hamster	1	5/10	50·0

per cent within the 7-day experiment period. Most of the embryos died within the first to third day following inoculation.

Whole blood from animals with solid tumors that readily undergo widespread metastasis was also extremely toxic and killed a large percentage of inoculated embryos. Plasma of animals with leukemia or metastasizing solid tumors was for the most part, less toxic in eggs than was the whole blood (Table 4).

These studies have also revealed that normal tissues and tumor tissues, *per se*, have never produced marked lethality in chick embryos.

In order to investigate the effect on chick embryos of blood of animals with alterations in normal physiology, blood from pregnant mice and hamsters was assayed. Blood of normal females, pregnant animals, and post-partum animals was quite innocuous in eggs, and embryo mortality was within the non-toxic range (Table 5).

In further studies, it was observed that blood of rodents with inflammatory lesions and granulomas produced by irritants, such as talc,

Table 4. *Lethal effect on chick embryos inoculated with blood from animals bearing metastasizing tumors*

Tumor	Host	No. of hosts assayed	No. of embryos dead/No. of embryos implanted	Dead (%)
Sarcoma T-241 (Lewis)	C57BL/6 mouse	17	72/82	87·8
AtTa adrenotropic pituitary tumor	LAF_1 mouse	6	23/29	79·3
Sarcoma HAS-1	Syrian hamster	8	90/119	75·6
Sarcoma AHT	Syrian hamster	2	10/18	55·5

Table 5. *Effect of blood of pregnancy on mortality of chick embryos*

Animal	No. of animals assayed	No. of embryos dead/No. of embryos implanted	Dead (%)
Swiss mouse			
Normal female	4	0/20	0·0
Pregnant	20	11/102	10·7
Post partum female (1–2 weeks)	12	3/58	5·2
Syrian hamster			
Normal female	5	3/26	11·5
Pregnant	14	15/72	20·8
Post partum female (1–2 weeks)	5	5/40	12·5

and with benign and malignant non-metastasizing tumors produced by chemical carcinogens was also non-toxic in eggs.

DISCUSSION

Other studies performed in these laboratories (to be reported elsewhere) utilizing different strains of White Leghorn eggs indicated that although there was consistently a significant difference in toxicity between blood from malignant and non-malignant sources, different strains of embryos responded differently in degree of toxicity. The Thompson strain yielding the greatest degree of sensitivity and most reproducible results was selected for the present investigation, as well as for those previously reported [1, 2].

The presence of a factor toxic to chick embryos has been demonstrated, not only in human whole blood and plasma, but also in whole blood and to a lesser degree in the plasma of animals with disseminated neoplasms. Observations by several other investigators agree and confirm in part these observations [4–9]. This factor is either not present or does not express itself in normal blood. The fact that the disease is disseminated in animals appears to be most significant, since little toxicity occurs in eggs inoculated with blood of animals with non-metastasizing tumors.

Whether the tumors are transplanted, spontaneous or induced apparently has little bearing on the toxic factor. Within the design of the present experiments, blood of animals with alterations in normal physiology, such as pregnancy, or with experimentally produced lesions was found also to be non-toxic in eggs.

The nature of the factor, mechanisms of its activity and the specificity of toxicity are currently under investigation.

That the nature of this factor is subcellular was suggested by earlier studies in these laboratories when marked toxicity was observed following the inoculation of fractions and ultrafiltrates of fractions of human leukemic leukocytes [2]. Further evidence was acquired when it was shown in these and other laboratories using different techniques [5, 9] that the toxic factor in human leukemic blood can be transmitted in eggs by serial passage through the use of ultrafiltrates. In experimentation in progress, ultrafiltrates prepared from embryos (organs and tissues) inoculated with blood from three cases of human leukemia and from five cases of AK4 leukemia, indigenous to AKR mice produced a high rate of lethality and marked pathological changes. These ultrafiltrates have been serially transmitted in eggs with retention of toxicity through eleven and six passages respectively.

Cytological, histological and biochemical studies are being performed on embryos and chickens surviving exposure to the toxic factor in order to investigate its mechanisms of activity. Preliminary results thus far indicate that in addition to its lethality, this factor also produces pathological changes; such as: severe hepatic damage, interference in hatching, inhibition of growth and sexual development, qualitative and quantitative blood cellular alteration, and depression in hemoglobin production [10, 11].

The specificity of this factor is being analyzed and evaluated in an expanded assay program involving the use of blood samples from 7000 patients and from 1000 animals. Results of these investigations await completion before being reported in detail.

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RÉSUMÉ

Le sang d'animaux porteurs de tumeurs solides métastasiantes de leucémies et de lymphomes provoque, chez les embryons de poulet, une mortalité élevée (40–100 *pour cent au 7e jour*), *lorsqu'il est injecté sur la membrane chorio-allantoïdienne. Au contraire, du sang provenant d'animaux normaux, d'animaux porteurs de tumeurs non métastasiantes, de lésions inflammatoires ou de granulomes, du sang d'animaux dont la physiologie normale est ultérée, injecté dans les mêmes conditions, est relativement inoffensif et ne provoque qu'un raible pourcentage de décès* (0–30 *pour cent*), *chez les embryons de poulet.*

SUMMARY

Blood from animals bearing solid metastasizing tumors, leukemias, and lymphomas produced a high rate of mortality of chick embryos (40–100 *per cent*) *within 7 days*

when inoculated onto the chorioallantoic membrane. In contrast, similarly inoculated blood from normal animals, those bearing tumors which do not metastasize, those with inflammatory lesions and granulomas and those with alterations in normal physiology was relatively innocuous and caused only a low percentage (0–30 *per cent*) *of death of chick embryos.*

ZUSAMMENFASSUNG

Blut, welches von mit metastasierenden soliden Tumoren oder mit Leukämien oder mit Lymphomen behafteten Tieren entnommen war, verursachte eine hohe Mortalität (40–100 *Prozent*) *von Hühnerembryonen innerhalb von sieben Tagen, wenn es auf die Chorioallantoismembrane überimpft wurde. Im Gegensatz hierzu erzeugte ebenso überimpftes Blut, welches entweder von normalen Tieren oder von solchen mit entzündlichen Prozessen und Granulomen oder von solchen mit Alterationen der normalen Physiologiestammte, nur relativ gutartige Veränderungen und nur eine Mortalität von* 0–30 *Prozent.*

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Testicular Leydig Cell Tumourigenesis by Diethylstilbestrol in the BALB/c Mouse

Histologic and Histochemical Study

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INTRODUCTION

THE BIOLOGIC and morphologic properties of interstitial cell testicular tumours developing following various experimental procedures in BALB/c and other strains of mice have been previously reported by numerous authors [1–9]. Most of these reports are indirectly dealing with the functional activity of these tumours by studying the weight changes and the histology of the accessory sex organs, i.e. the seminal vesicles–coagulating gland complex. However the hormonal production of the testicular interstitial cell tumours in mice has been proved by biochemical methods [10–15].

The present investigation has been undertaken in order to study: (a) the weight changes of testes and adrenals during oestrogen treatment; (b) the histologic changes induced by the treatment in the testes; (c) the histology of the secondary sex organs and of the adrenal cortex in the course of the experiment; (d) the alterations of spermatogenesis during tumourigenesis; (e) the histo-enzymology of the hyperplastic and neoplastic testicular interstitial-cell tissue and of the adrenal, and the histochemistry of lipids and pigments in the same tissues.

MATERIAL AND METHODS

The experiment was carried out on BALB/c male mice of the colony of this laboratory. This colony originated from BALB/c mice obtained in 1962 from the National Institute of Health, Bethesda, Maryland, U.S.A., through the courtesy of Dr. M. K. Deringer. Since then they were bred randomly in this laboratory. The animals were housed in groups of six to eight in plastic cages on wood shavings, fed Randoin and Causeret mouse diet in pellets, with water *ad libitum*. A group of them at the age of 6–8 weeks, received subcutaneous implantations in the right flank of diethylstilbestrol-cholesterol pellets. Farmitalia (Milano, Italy) kindly supplied the pellets which were prepared according to a technique proposed by Shimkin [16]; each pellet weighed approximately 5 mg and contained 25 per cent of diethylstilbestrol in cholesterol. The pellets were inserted by means of a 12-gauge trocar. A second group of untreated male mice was used as control. All animals were examined every week and the body weights recorded. Ten treated and ten untreated control animals were killed by cervical dislocation at the 30th, 60th, 90th, 120th, 150th and 180th day after pellet implantation and the body weight, the weight of the testes and adrenals were recorded. All killed animals were autopsied and sections of one testis, one adrenal, seminal vesicles–coagulating gland complex were kept for histologic examination. The tissues were fixed in Bouin's fluid embedded in paraffin and stained with haematoxylin and eosin. The second testis and adrenal were rapidly frozen in a dry ice–acetone mixture to −70°C and cut at 10μ with a cryostat (−20°C) for cytochemistry.

Unfixed cryostat sections were treated with cold acetone (4°C) for lipid extraction, and subsequently incubated for demonstration of the Δ^5—3β-hydroxysteroid dehydrogenase (3β-HSD) according to the method proposed by Levy *et al.* [17]. The following substrates were used: dehydroepiandrosterone (DHA), pregnenolone and 17α-hydroxypregnenolone (Light Co.); Nitro BT was used as the final electron acceptor. A control medium containing all reagents but not the substrates was employed. Some sections were incubated for demonstration of glucose-6-phosphate dehydrogenase (G-6-PD) and NADPH diaphorase [18].

An additional experiment was carried out to study the lipid pigments both in testis and adrenal cortex. A number of stilbestrol treated and control animals were sacrificed at the previously described periods. The testes and adrenals were fixed in formaldehyde-calcium and subsequently embedded in gelatin–sorbitol according to Turolla *et al.* [19]. The embeddings were rapidly frozen in dry ice to −70°C and cut at 10μ with a cryostat (−20°C). Cryostat sections were submitted to the following procedures for histochemical identification of the pigments and lipids: (1) iodine–iodide reaction for carotenoids; (2) bleaching with performic acid for melanins; (3) Sudan black B reaction for lipopigments; (4) acid fastness for "ceroid"; (5) Blue Nile reaction for chromolipoids; (6) Performic acid–Schiff reaction for C = C groups; (7) PAS reaction; (8) O_2-Schiff reaction (pseudoplasmal); (9) M-NADI reaction for fatty peroxides; (10) Schmorl test for lipofuscins; (11) Perls reaction for iron (haemosiderin).

RESULTS

A. *Weight changes and gross findings*

The main macroscopic results of the investigation are summarized in Table 1. In the oestrogen treated animals there was a marked decrease of the mean body weight on the 30th day after the pellet implantation; starting from the 90th and up to the 180th day a progressive weight gain was observed. As far as the testis weight is concerned, a clear cut weight increase, both absolute and relative, was observed only in treated animals with evidence of well developed interstitial-cell hyperplasia or tumour, i.e. 120, 150 and 180 days after the pellet implantation.

A significant increase of the mean and percentage weight of the adrenals was observed in all the treated groups.

Table 1. *Animals developing interstitial cell tumours (ICT) in control and diethylstilbestrol treated BALB/c mice serially killed*

Body, testes and adrenals weights in control and diethylstilbestrol treated BALB/c mice serially killed.

Control

No. of mice	Body weight (g) In.	Fin.	Weight changes [%]	Mean weight of testis (mg)	(% b.w.)	Mean weight of adrenals (mg)	(% b.w.)	ICT bearing animals
10	16·50	26·00	+36·54	135·0	519·2	5·3	22·03	0
10	16·40	28·50	+42·46	148·8	522·1	6·3	22·10	0
10	16·25	24·00	+32·30	152·0	633·3	4·1	17·08	0
10	18·25	27·30	+33·16	169·9	622·3	4·3	15·75	0
10	16·16	25·00	+35·36	136·9	547·6	5·7	22·80	0
10	16·00	24·50	+34·70	133·5	544·8	4·3	17·55	0

Diethylstilbestrol

Days of treatment	No. of mice	Body weight (g) In.	Fin.	Weight changes	Mean weight of testis (mg)	(% b.w.)	Mean weight of adrenals (mg)	(% b.w.)	ICT bearing animals
30	10	19·80	16·20	−18·20	115·3	711·7	7·2	44·44	0
60	10	20·16	20·12	−0·20	119·1	592·5	7·8	38·80	0
90	10	17·50	19·40	+9·80	118·9	612·8	8·7	44·84	0
120	10	17·70	22·76	+22·03	204·6	901·3	9·5	41·85	2
150	10	19·21	25·20	+21·00	241·5	958·3	10·4	41·26	7
180	10	19·00	23·66	+19·50	284·0	1203·3	10·2	43·22	8

Grossly the testes of animals killed from the 60th day to the 90th day after pellet implantation showed on their surface many small yellow spots, and 120–180 days after implantation, one or more yellow-greyish nodules clearly separated by non-involved testicular parenchyma.

The number of tumours was increasing with the duration of the treatment: we found two interstitial cell tumour (ICT) bearing animals on the 120th day, seven ICT bearing animals on the 150th day, and finally eight ICT bearing animals on the 180th day.

In most treated animals killed between 60 and 180 days, the vesicle—coagulating gland complex was enlarged and filled with a dense yellow fluid.

B. *Histologic findings*

1. *Testis*—The most striking histologic changes observed in the testes after pellet implantation occurred in the interstitial cell system and in the seminal epithelium. Of ten treated mice killed at the 30th day only one showed a moderate degree of interstitial-cell hyperplasia and four a very low one.

As early as 60 days after the beginning of the experiment such hyperplasia could be histologically described as initially composed of interstitial cells with large and irregular nuclei and abundant, vacuolated cytoplasm. These changes were usually found in the majority of our treated animals killed from the 60th to the 90th day of experiment; they progressed to the presence of many large binucleated cells. Further progression toward a tumour was generally preceded by the formation of larger nodules composed of enlarged Leydig cells between the seminiferous tubules (Fig. 3). The hyperplastic interstitial cells areas, always surrounded by seminiferous tubules showing various degrees of cellular regression, progressed through the formation of larger nodules in to a well developed tumour (Fig. 4). In interstitial tumours, that could be observed from the 120th day, many cellular types were found: large vacuolated cells together with granular cells and small elements with small hyperchromatic nuclei. Pigment deposition brown in colour was frequently observed in groups of hyperplastic or tumoural Leydig cells; such intra-cytoplasmic material appeared to be composed of coarse and partially clumped irregular granules.

In hyperplastic Leydig tissue only cells of the first two described types were found. This statement is in agreement with the data reported by Hooker and Pfeiffer [4] and many others. In the present study we restricted the term "tumour" to nodular areas showing an adenomatous pattern arrangement of the hyperplastic Leydig cells and, especially, evidence of structural disorder and marked regression of the surrounding seminiferous tubules. Previously [3] the term tumour was given to masses of a size double that of a normal testis; other authors [5] considered as tumours nodular areas occupying one third to one half of the testis. The interstitial cell tumours were composed of solid sheets or cords of large, polygonal cells supported by a sparse, thin connective stroma. The Leydig cells were often arranged in adenomatous pattern around endothelial-lined blood containing spaces. In larger tumours, haemorrhages were frequently seen. It is note-worthy to say that in these tumours the mitotic figures are rarely observed. Metastases were never found.

In comparison with the control testis (Fig. 1), the histologic changes observed in the seminiferous tubules during the whole duration of the experiment could be classified into three phases: (a) 30 days after the beginning of the experiment a marked regression of the seminiferous epithelium with presence of numerous giant cells free in the lumina was observed as well as numerous degenerated seminal cells (Fig. 2); (b) 60 and 90 days after implantation there was a partial functional revival of the seminal activity with appearance of spermatids and disappearance of giant cells (Fig. 3); (c) at the 120th and 180th day of the experiment, when the Leydig cells hyperplasia was more evident and the interstitial cell tumours well developed, most of the seminiferous tubules showed complete regression of the spermiogenesis with only one or a few layers of germinal cells.

2. *Seminal vesicles-coagulating gland complex* (Figs. 5 and 6)—Shortly after implantation, we found an extensive squamous metaplasia of the coagulating gland epithelium (Figs. 7–10) and a mild hypotrophy of the seminal vesicles together with leucocyte infiltration. Later, i.e. on the 150th and 180th day, a persistent squamous metaplasia of the coagulating epithelium with evidence of functional revival of the seminal vesicles epithelium was found (Figs. 8–10).

3. *Adrenals* — As previously mentioned the adrenals of the treated animals showed a significant weight gain during the whole duration of the experiment. The histologic pattern

was characterized by clear modifications in the inner zones of the cortex. In the cytoplasm of the cells of the reticular zone there was a deposition of a granular pigmented material very similar to that observed in Leydig cells. These pigmented cells rarely appeared as isolated and mononucleated; more frequently as multinucleated syncitial masses with pyknotic nuclei. This histologic feature was present throughout the entire observation period. It is noteworthy to say that in all our treated animals the juxtamedullary *x*-zone had completely disappeared regardless of the period of treatment. Except for the presence of some intra or subcapsular nodular areas of hyperplasia consisting of clear cells similar to those normally present in the fascicular zones, the outer zones of the cortex as well as the medulla were morphologically normal for the whole duration of the experiment. The outer areas of medulla were sometimes partially occupied by pigmented deposition. In addition we have frequently observed small foci of extramedullary hemopoiesis scattered in the outer cortical zone.

C. *Enzyme histochemistry of the testis*

1. *Control testis* — 3β-HSD. The most intense formazan deposition occurred in the Leydig cells at all observed ages when pregnenolone was used as substrate; a less intense reaction was obtained with DHA and a more weak reaction had been observed with a 17α-pregnenolone. The seminiferous tissue always showed negative results (Fig. 11) *G-6-PD and NADPH-diaphorase.* Intense formazan deposition was observed in the Leydig cells at any considered period. A weak activity was also detected in the seminiferous epithelium.

2. *Treated testis* — 3β-*HSD.* The histochemical results obtained with the above reported substrates in the treated testis are summarized as follow: the most intense formazan deposition was found in the testis incubated with pregnenolone and, to a lesser extent, with DHA. With 17α-hydroxypregnenolone a very weak positive reaction was found.

At the 30th day of treatment, the intensity of formazan deposition within the Leydig cells, with the different substrates, was similar to that observed in the control animals. The reaction was uniformly distributed in all the testicular interstitial tissue and was gradually increasing as interstitial hyperplasia became evident (Fig. 12). Cells filled with lipid pigments showed no enzymatic activity.

In the interstitial cell tumour tissues 3β-HSD activity could be demonstrated in nodular groups of cells (Fig. 13), sometimes showing a rise in activity, as compared to that found in interstitial hyperplasia. In some instances the interstitial tissue surrounding the tumour was found to be enzymatically inactive. *G-6-PD and NADPH-diaphorase.* The histochemical distribution of these oxidative enzymes did not significantly differ from the localization of 3β-HSD: however, it is noteworthy that the enzymes connected with the hexose monophosphate shunt gave a formazan deposition more intense than that observed for the 3β-HSD. In the pigmented cells these enzymatic activities were also lacking.

D. *Enzyme histochemistry of the adrenal cortex*

DHA was the only substrate giving a pink blue formazan deposition when incubated with the adrenal gland for the visualization of 3β-HSD. Therefore DHA was the substrate utilized for the study of the adrenal cortex.

1. *Control adrenal cortex* — 3β-*HSD* activity was localized by a heavy blue formazan deposition in the zona fasciculata and reticularis. Only a faintly positive reaction was observed in the zona glomerulosa. *G-6-PD and NADPH* diaphorase activities gave a fairly similar histochemical distribution.

2. *Treated adrenal cortex* — As the pigmented cells appear and collect in nodular sincytiums in the inner zones of the cortex, the juxtamedullary cortical zone was found to be enzyme-histochemically completely negative. The 3β-HSD, G-6-PD, and NADPH-diaphorase activities were clearly detected only in the fasciculata and, to a lesser extent, in the zona glomerulosa (Fig. 14). The observed intracapsular or subcapsular areas of nodular hyperplasia showed marked oxidative enzymatic activities.

Cells filled with pigments did not show any enzymatic activity.

E. *Histochemistry of the pigments and lipids*

1. *Testis* — As early as 30 days after the beginning of the treatment, yellow-brown granules were observed in the Leydig cells; 60 days after stilbestrol implantation the majority of the enlarged Leydig cells showed their cytoplasms filled with heavily pigmented

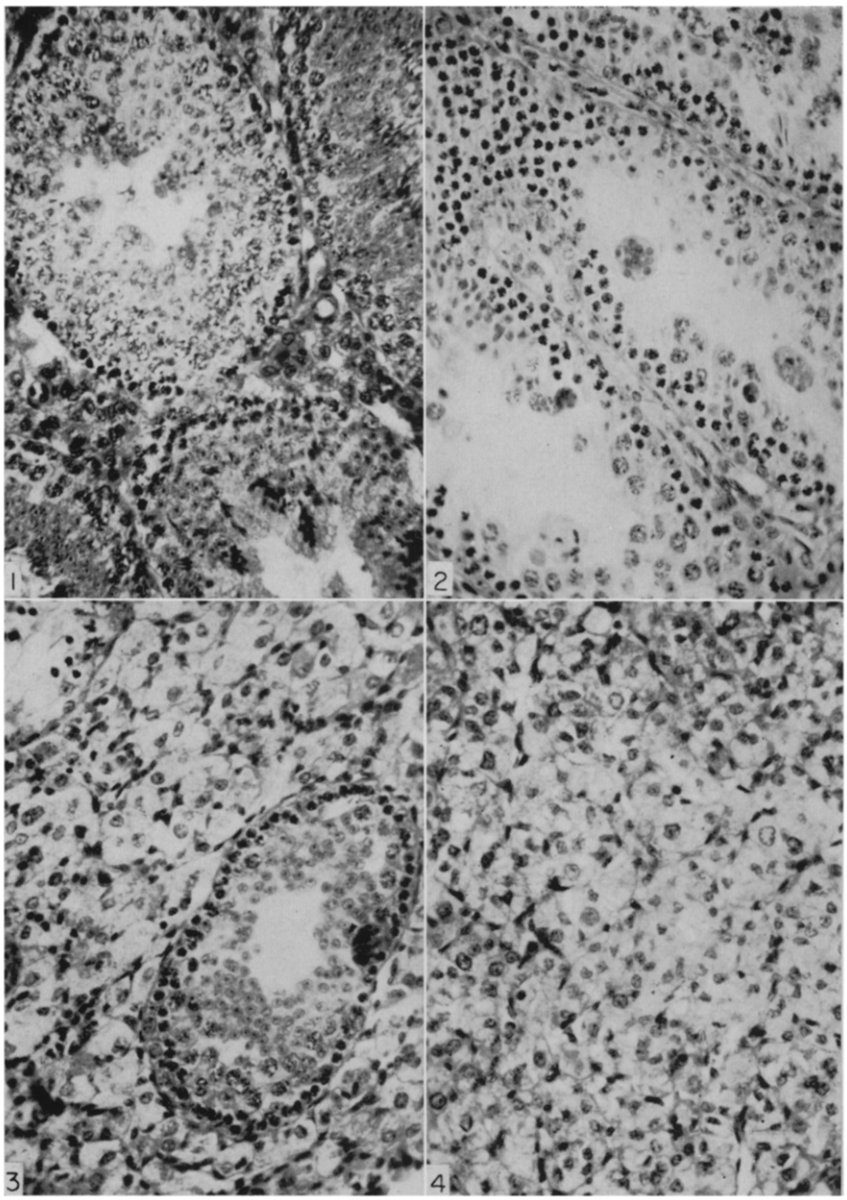

Fig. 1. *Testis of a control mouse. Haematoxylin and eosin stain.* × 256.

Fig. 2. *Testis of a* 30 *days treated mouse. Marked regression of the seminal epithelium and blocked spermiogenesis with giant cells. Haematoxylin and eosin stain.* × 256.

Fig. 3. *Testis of a* 90 *days treated mouse. Marked hyperplasia of the Leydig tissue; in the seminiferous tubules evidence of early spermatids. Haematoxylin and eosin stain.* × 256.

Fig. 4. *Testis of a* 150 *days treated mouse. Interstitial cell tumour. Haematoxylin and eosin stain.* × 256.

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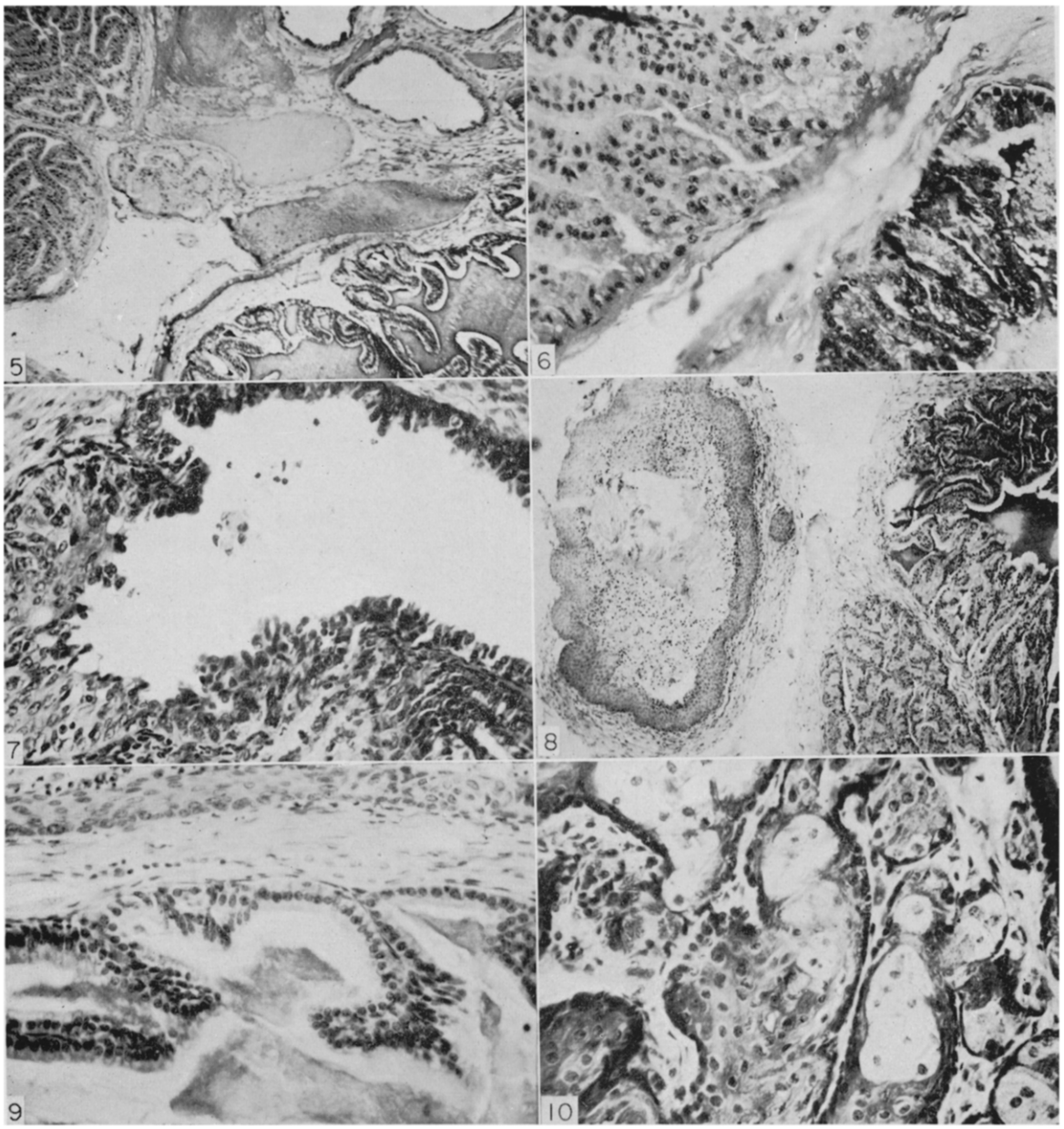


Fig. 5. *Seminal vesicles–coagulating complex of a control mouse. On the left upper corner the coagulating gland. On the right lower corner the vesicular gland. Haematoxylin and eosin stain.* × 64.

Fig. 6. *Same as Fig.* 5 *at higher magnification. On the left coagulating gland; on the right vesicular gland. Haematoxylin and eosin stain.* × 256.

Fig. 7. *Coagulating gland of a* 30 *days treated mouse. Initial squamous metaplasia. Haematoxylin and eosin stain.* × 256.

Fig. 8. *Seminal vesicle–coagulating gland complex of a* 180 *days treated mouse. On the left squamous metaplasia of the coagulating gland and on the right initial functional revival of the vesicular gland. Haematoxylin and eosin stain.* × 64.

Fig. 9. *Same as Fig.* 8 *at higher magnification; on the upper side squamous metaplasia of the coagulating gland and on the lower side functional revival of the vesicular gland. Haematoxylin and eosin stain.* × 256.

Fig. 10. *Coagulating gland of a* 150 *days treated mouse. Marked dyskeratosis of the epithelium. Haematoxylin and eosin stain.* × 256.

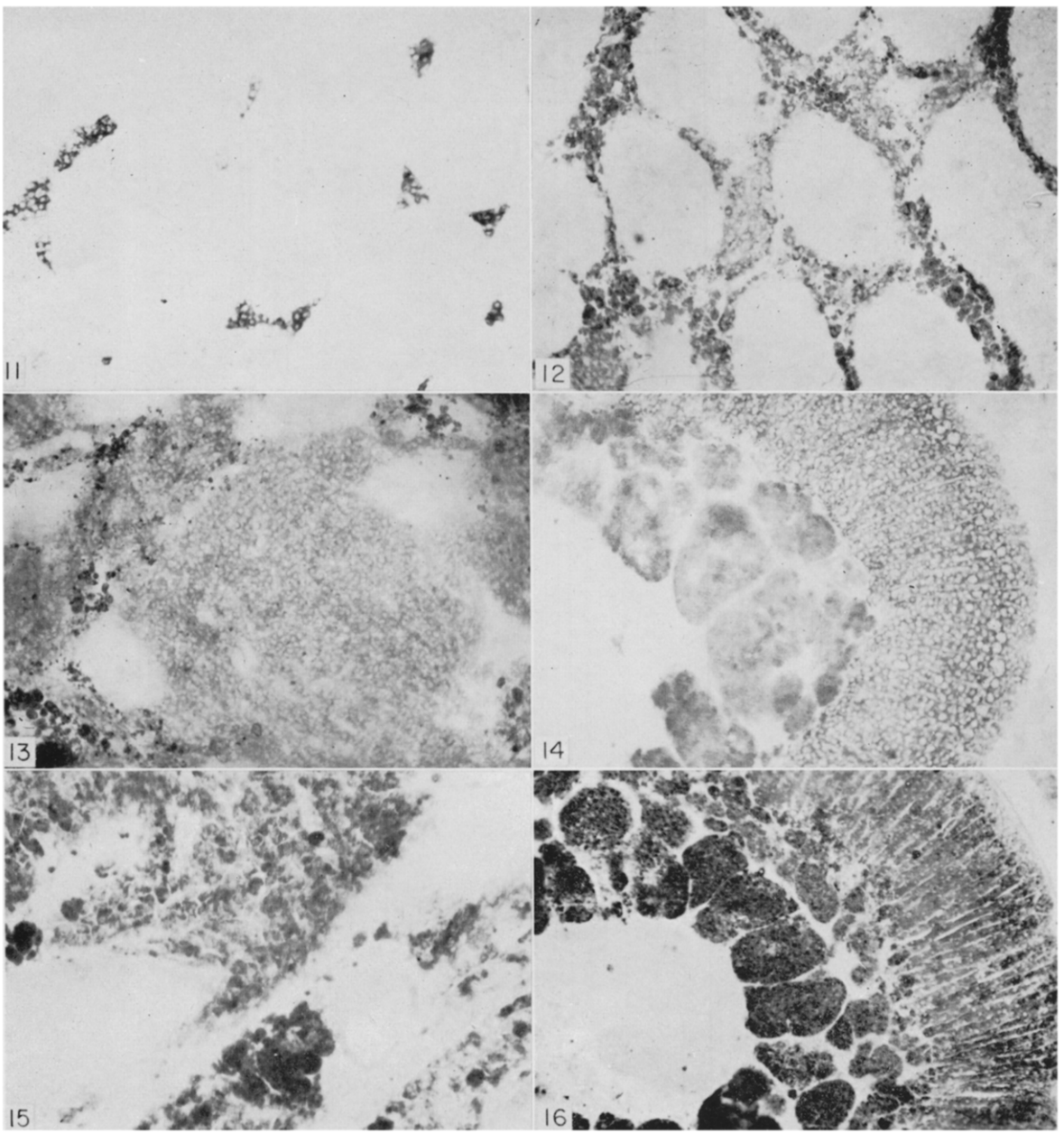


Fig. 11. *Testis of a control mouse.* 3β-*HSD; pregnenolone as substrate.* × 100.

Fig. 12. *Testis of a* 60 *days treated mouse. The hyperplastic Leydig tissue exhibits evidence of* 3β-*HSD activity; pregnenolone as substrate.* × 100.

Fig. 13. *Testis of a* 120 *days treated mouse. Small interstitial cell tumour showing moderate* 3β-*HSD activity; pregnenolone as substrate.* × 100.

Fig. 14. *Adrenal cortex of a* 120 *days treated mouse. The* 3β-*HSD activity is limited to the outer cortex. The inner cortex is filled with pigments. DHA as substrate.* × 100.

Fig. 15. *Testis of a* 120 *days treated mouse. Ceroid deposition in interstitial cells stained dark blue by Nile blue reaction.* × 256.

Fig. 16. *Adrenal cortex of a* 150 *days treated mouse. Ceroid deposition in the reticular zone stained dark blue by Nile blue reaction; the fasciculata is also pale blue stained.* × 100.

granules which increased in the animals sacrificed later. Pigmented Leydig cells were often observed to coalesce into large multinucleated giant cells with cytoplasm completely filled by pigmented droplets. Large areas of pigmented tissue together with Leydig cells showing degenerative or atrophic changes were found inside the tumour of the animals sacrificed 180 days after implantation.

The results of the various histochemical staining reactions used in this study can be summarized as follows: (1) the pigment was only partially soluble in the liposolvents; (2) Sudan black B reaction was positive in almost all the cells with the exception of few of them; (3) Blue Nile reaction was strongly positive (Fig. 15); (4) acid fastness was positive; (5) performic acid–Schiff reaction was positive; (6) PAS reaction was positive in almost all the cases; (7) M-NADI reaction was irregularly fairly positive; (8) Perls and O_2-Schiff reactions and Schmorl test were negative.

The pigment free Leydig cells were partly positive to the Sudan Black B and Blue Nile reactions in proportion to their content of neutral fats and phospholipids; they were also moderately positive for the performic acid Schiff and O_2-Schiff reactions.

2. *Adrenal cortex* — The histochemical properties of the pigment found in the reticular zone of the adrenal cortex of the treated animals, were similar to those described for the testis (Fig. 16) with the exception of a moderately positive focal acid fastness, a negative M-NADI reaction and an always positive, though weak, Schmorl test.

Blue Nile and Sudan black B positive neutral fats, and phospholipids positive to O_2-Schiff and performic acid-Schiff methods, were additionally observed in the glomerular and fascicular zones.

As previously pointed out the adrenal cortex cells as well as the Leydig cells containing pigments, appeared to be always negative for the enzymatic activities studied here.

DISCUSSION

As far as the body and testes weight changes in diethylstilbestrol treated BALB/c mice are concerned, we found a high testicular relative weight in the treated group killed on the 30th day. In our opinion this fact is due to the body weight loss secondary to the stilbestrol pellet implantation, since no significant interstitial cell hyperplasia was observed. In fact the final body weight as well as the body weight changes recorded for our treated groups were constantly less pronounced than the corresponding values of the control groups. As previously reported [20] we observed a loss of body weight particularly marked in the group sacrificed at the 30th day and to a lesser extent at the 60th day. The loss of body weight after oestrogen treatment might be attributed to the reduction of food uptake in oestrogen treated animals [21].

Our results appear to differ from those presented by other authors [9], who showed an early increment of the testicular weight of the treated series becoming particularly evident on the 120th, 150th and 180th day of treatment and a higher incidence of Leydig tumours with a shortening of their latent period.

A striking finding in our treated animals was a marked adrenal enlargement. It has been reported [20] that in the BALB/c mouse, adrenal hypertrophy constantly occurs after treatment with a natural oestrogen, for instance oestroadiol. Nevertheless this experiment and the reported data cannot be completely compared with ours since they were obtained on gonadectomized animals treated with oestradiol only 20 and 30 days. As far as the action of natural and synthetic oestrogens on the adrenal cortex is concerned, different opinions are reported [22–33].

Our histologic observations during the whole duration of the experiment indicate that the Leydig cells, the seminal vesicles-coagulating gland complex and the adrenal cortex are highly susceptible to the action of a pellet of diethylstilbestrol. In agreement with previously reported observations [6–9] we found the BALB/c mouse highly susceptible to the induction of Leydig tumours following diethylstilbestrol treatment. The appearance of these tumours is generally preceded by a period in which the Leydig cell system shows a marked hyperplasia of its elements. In the great majority of the treated animals a significant degree of Leydig cells hyperplasia was found within the 60th day. The presence of many large binucleated cells sometimes brown in colour together with hyperplastic testicular interstitial cells was interpreted as altered Leydig cells rather than phagocytes [5].

The histologic findings of the seminiferous tubules in our treated animals are not in complete agreement with previously reported observations [9] in which an almost complete preservation of spermatogenesis in oestrogen treated BALB/c mice was described. Our results suggest that during the first phase of the

interstitial cell tumourigenesis by diethylstilboestrol, the seminiferous system is completely under the influence of the exogenous oestrogen. In a second phase the progressively developing interstitial hyperplasia with concomitant androgenic secretion may be able to act as a protective mechanism against oestrogen indirectly determining a partial revival of the seminal activity. Finally, in testes containing tumours, the marked atrophy of the seminiferous tubules could be interpreted as a manifestation of an exhaustion of the protective mechanism against the oestrogen [9] as well as caused by a mechanical compression of the tubules by the tumour. In fact we have found that the functional exhaustion of the interstitial cells could be demonstrated by the loss of the enzymatic activities related to the steroid production.

The extensive squamous metaplasia of the coagulating gland epithelium has been reported before [15]. Our study has also shown that, while the oestrogen induced hypotrophy of the seminal vesicles has a tendency to disappear with evidence of functional revival under the effect of the hyperplastic interstitial cells androgenic secretion, the coagulating gland epithelium squamous metaplasia is a constant finding regardless of the time of death.

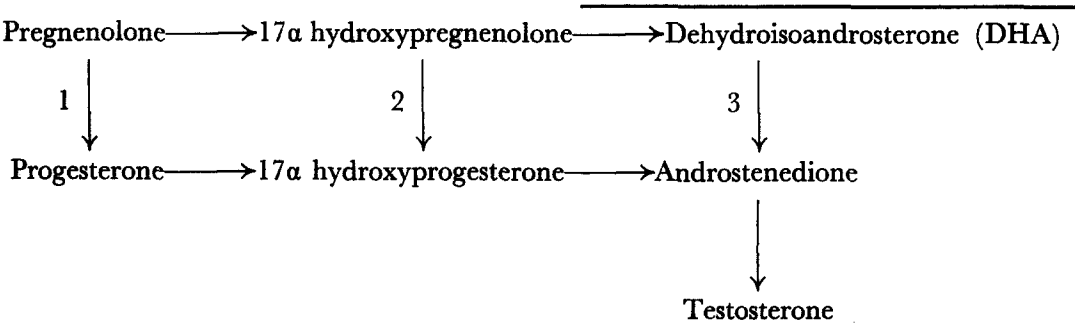
In order to directly ascertain if the testicular hyperplastic or tumoral interstitial cells are steroids secreting elements, we have histochemically detected 3β-hydroxysteroid dehydrogenase (3β-HSD), glucose-6-phosphate dehydrogenase (G-6-PD) and nictotinamide-adenin-disphosphate reduced diaphorase (NADPH diaphorase) activities. The 3β-HSD oxidizes the Δ^5-3β-hydroxylic group to Δ^4-3oxo-group with the participation of a $\Delta^5-\Delta^4$ isomerase. The Δ^4-3oxo-group is characteristic of all biological active steroid hormones.

[34, 35], in which various steroid substrates were used for the 3β-HSD detection.

The G-6-PD, "via" oxidation of G-6-P in the hexose-monophosphate "shunt," provides NADPH, which is thought to be essential for a number of hydroxylations in the synthesis of various steroid hormones [36, 37]. Finally, the NADPH diaphorase catalyses the transfer of the H^+ from the coenzyme to the tetrazolium salt.

In our treated animals killed at the 60th and 90th day we found a particularly high oxidative enzymatic activity in all the interstitial cells. Starting from the 120th day and after, we have noted the appearance of numerous Leydig cells containing lipid pigment in the hyperplastic interstitial cell areas as well as in the neoplastic nodules. In the meantime the pigment filled cells were gradually loosing the enzymatic activities under investigation and these were also irregularly distributed.

The more marked formazan deposition observed with pregnenolone as substrate might suggest that "pregnenolone-3β-HSD" is the more active enzyme in metabolizing the substrate not only in normal mouse testes [34], but also in hyperplastic and neoplastic conditions. The presence of oxidative enzyme activities in neoplastic Leydig tissues might suggest that the interstitial tumoral cell is histochemically quite similar to the normal cell except in the cases in which there is pigment deposition. It appears that the 3β-HSD is essential at an early step of the steroid hormone biosynthesis. For this reason we cannot indicate by histochemical means if the Leydig cells secrete androgens, oestrogens or C_{21} corticoids. Biochemical findings [15] have shown in diethylstilbestrol induced Leydig cell tumours an inhibition of enzymatic activities at the end of the biosynthetic pathway of the testosterone (17α-



Suggested pathway for the biosynthesis of testosterone; 3β-HSD may act at sites, 1, 2, 3 (*partly modified from Huseby* et al. [15]).

We have used as substrates DHA, pregnenolone and 17α hydroxy pregnenolone in view of the different results recently reported

hydroxylase and side chain-splitting enzyme). Our histochemical results suggest, in agreement with the biochemical data [15], that the hyperplastic and tumoural Leydig cell retains 3β-HSD activity even when other steroid biosynthetic enzyme systems diminish or are lost

completely. Finally, it would seem most likely that, even when the interstitial testicular cell appears cytologically abnormal, some oxidative enzymatic activities suggesting a potentially active hormonal synthesis, are still present.

Pigmented lipids in the testicular interstitial cells of diethylstilbestrol treated mice have been previously reported [3, 4, 38, 39]. Our histochemical data might suggest a suitable interpretation as far as its chemical structure and its significance is concerned. In the actively proliferating Leydig cells, the pigment, interpreted as a chromolipoid, has shown fairly uniform histochemical properties corresponding to those of "ceroid," which is considered as an intermediate step in the metabolic system transforming the unsaturated fatty acids into lipofuscins, through peroxidation and polymerization processes [40, 41]. The positivity of the Sudan black B and blue Nile reactions and especially the frankly positive acid fastness are strong support for this hypothesis. The initial phases of the ceroid synthesis represented by fat peroxides, are indicated by the observation of an irregularly fairly positive M-NADI reaction. The lack in positivity of the Schmorl test indicates that the pigment, in our treated material, does not reach the end of its biosynthetic pathway, i.e. the stage of lipofuscin. The finding of various intermediate stages of peroxidation-polymerization processes in the same specimen, strongly supports a rapid evolution of the ceroidogenesis.

The deposition of considerable amount of ceroid pigment in the inner adrenocortical region of the oestrogen treated animals, has been previously published but its significance is still unknown. [20, 42, 43]

The histochemical properties of the pigment in the adrenal cortex of our treated animals, mostly the negative M-NADI reaction, the fairly positive Schmorl test and the moderately positive focal acid fastness, indicate that in this tissue, the pigment reaches a more advanced step of its biosynthesis than it does in the testes.

Our observations on the enzyme histochemistry of the adrenal cortex show that the cells in which ceroid deposition occurs are negative for the detection of some enzyme activities related to steroidogenesis. In fact these cells are devoid of 3β-HSD activity which is an essential step in the biosynthesis of all the biologically active steroids. Moreover, the absence in the ceroid filled-cells of other enzymatic activities indirectly connected with steroid biogenesis, i.e. G-6-PD and NADPH diaphorase, suggests a block of the steroid hydroxylation mechanism. The absence of a well defined *x*-zone in our treated animals may be related to their post-puberal age. The presence of some intra or subcapsular areas of nodular hyperplasia in the outer cortex of some treated animals has not been considered as induced by the treatment, the same picture having been observed in control animals.

In the adrenal as well as in the testes it is likely that the ceroid formation should be preceded by an accumulation of lipids associated with an absolute or relative diminution of their metabolic rate normally leading, through β-oxydation of fatty acids, to the synthesis of active acetate and subsequently of steroids. The presence of unsaturated highly oxidizable fatty acids, determines the formation of chromolipoids, i.e. ceroids, through a peroxidation followed by polymerization [41].

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RÉSUMÉ

Le présent travail concerne une étude sur les modifications macroscopiques, histologiques et histochimiques au niveau des testicules et des glandes surrénales de souris BALB/c, après implantation sous-cutanée d'une tablette de diéthylstilboestrol. Les animaux furent tués 30, 60, 90, 120, 150 *et* 180 *jours après l'implantation. On notait d'abord une importante perte de poids corporel, une augmentation de poids des testicules apparaissant au* 90*e jour, et une augmentation constante du poids des surrénales, dès le début de l'expérience. L'hyperplasie des cellules de Leydig était observée, chez les animaux traités, à partir du* 60*e jour, et les tumeurs des cellules de Leydig à partir du* 120*e jour. Une diminution importante des spermatocytes était visible peu de temps après l'implantation; ultérieurment une recrudescence partielle de l'activité séminale était observée, pour arriver finalement à une régression de la spermatogénèse. L'épithélium des glandes coagulantes montrait une métaplasie épidermoïde persistante. L'atrophie initiale de l'épithélium des vésicules séminales faisait place à des signes de réapparition d'activité dans les stades plus tardifs de l'expérience.*

L'étude histochimique des testicules et des surrénales a apporté des informations utiles sur l'activité des enzymes participant à la synthèse des stéroïdes, entre autres la 3β-hydroxystéroïdedéshydrogénase, la glucose-6-phosphate-déshydrogénase et le NADPH diaphorase. Les enzymes furent mises en évidence dans les tissus normaux, dans les tissus hyperplasiés et dans les tumeurs des cellules de Leydig. Une absence d'activité enzymatique fut observée dans les zones internes du cortex surrénal ainsi que dans certaines cellules de Leydig où l'on trouvait fréquemment des dépôts céroïdes.

SUMMARY

The present study is dealing with an investigation on gross, histologic and histochemical changes in testes and adrenal cortex of BALB/c mice after subcutaneous implantation of a diethylstilbestrol pellet. The animals were serially killed 30, 60, 90, 120, 150 and 180 *days after the implantation. They showed an initial marked loss of body weight, an increased testicular weight starting from the* 90*th day and a constant adrenal weight increase from the beginning of the experiment. Leydig cell hyperplasia was observed in the treated groups starting from the* 60*th day, and Leydig cell tumours from the* 120*th day. A marked regression of the spermatogenic elements was observed shortly after implantation; subsequently a partial revival of the seminal activity with final regression of spermiogenesis was found. The seminal vesicles–coagulating gland complex showed a persistent squamous metaplasia of the coagulation epithelium and evidence of initial atrophy of the seminal vesicles epithelium showing a functional revival in the later stages of the experiment.*

The histochemical study carried out on testes and adrenal cortex gave useful information on some enzyme activities connected with steroidogenesis, i.e. 3β*-hydroxysteroid dehydrogenase, glucose-*6*-phosphate dehydrogenase and NADPH diaphorase. The demonstration of the reported enzymes was obtained within normal, hyperplastic and tumoural Leydig tissues. A lack of enzyme activities was found in the inner zones of the adrenal cortex as well as in some Leydig cells where ceroid deposition was frequently found.*

ZUSAMMENFASSUNG

Die vorliegende Arbeit befaßt sich mit einer Untersuchung über die makroskopischen, histologischen und histochemischen Veränderungen in den Hoden und in der Nebennierenrinde von BABL/c/Mäusen im Anschluß an die subkutane Implantation einer Diäthylstilböstrol-Tablette. Die Tiere wurden gruppenweise, bezw. 30, 60, 90, 120 *und* 180 *Tage nach der Implantation getötet. Sie zeigten einen frühzeitigen, bedeutenden Gewichtsverlust, einen Anstieg des Hodengewichtes vom* 90. *Tag an und eine konstante Gewichtszunahme der Nebennieren vom Beginn der Experimente an. Eine Hyperplasie der Leydigischen Zellen wurde in den behandelten Tieren vom* 60. *Tag an beobachtet und Tumoren derselben vom* 120. *Tag. an. Eine bedeutende Rückbildung der spermatogenen Zellen wurde bald nach der Implantation bemerkt; etwas später kam es zu einer teilweisen Wiederbelebung der Samenbildung, aber nachher stellte sich ein neuerlicher Rückgang derselben ein. Die Samenbläschen und die Koagulationsdrüsen zeigten eine dauernde squamöse Metaplasie des Epitheliums der Koagulationsdrüsen und eine anfängliche Atrophie, später eine funktionelle Wiederbelebung des Epitheliums der Samenbläschen.*

Die histochemischen Untersuchungen der Hoden und der Nebennieren ergaben nützliche Hinweise über Enzymaktivitäten im Zusammenhang mit der Steroidbildung, z.B. über die 3-β*-Hydroxysteroid-dehydrogenase, die Glukose-*6*-phosphatdehydrogenase und über die NADPH-diaphorase. Die Darstellung der genannten Enzyme gelang in normalen, in hyperplastischen und in tumoralen Leydigischen Zellen. Ein Mangel an Enzymaktivität wurde in den inneren Zonen der Nebennierenrinde und in einigen Leydigischen Zellen mit Ceroidablagerung gefunden.*

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The Properties of Cell Clones Derived from a Polyoma Induced Mouse Tumour

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INTRODUCTION

TRANSPLANTABLE tumours induced by Mill Hill polyoma virus in mice, rats and hamsters, show the presence of virus but the amount is less than would be expected if every cell contained one infectious particle. In some tumours this infectivity can only be demonstrated in mouse kidney cultures, which have been shown to be more sensitive to polyoma infection than mouse embryo fibroblasts [1].

Cells from a polyoma-induced C3H mouse fibrosarcoma were cloned to see whether infectivity could be demonstrated in colonies arising from single cells. Two clones were obtained differing in morphological and biological characteristics but possessing a common antigen. Their properties will be discussed in further detail.

MATERIALS AND METHODS

Animals — C3H mice are bred by brother sister mating.

Tissue culture — Primary monolayer tissue cultures from 14-day-old C3H embryos [2] and kidney cultures from 5-day-old C3H mice [3] are kept static at 37° for 2 days and then rolled at 37°C for 3 days before use. The medium is changed twice weekly and consists of:

10% calf serum.
0·5% lactalbumin hydrolystate.
89·5% Hanks solution plus antibiotics.

Virus stock — This stock is obtained from a tissue culture line after treatment with receptor-destroying enzyme (R.D.E.). 0·1 ml contains 10^7 tissue culture infectious doses (T.C.I.D.) for mouse kidney (M.K.) cultures.

Haemagglutination — Twofold serial dilutions of virus in phosphate buffered saline (P.B.S.) are treated with 3×10^7 guinea pig red blood cells, in the same medium, at 4°C. The end point is taken to be the last dilution preceding incomplete haemagglutination [4].

Haemagglutination inhibition test — Twofold serial dilutions of inactivated sera are mixed with two to eight haemagglutinating doses of virus at room temperature for 2 hr and then left at 4°C with 3×10^7 guinea pig red blood cells. The end point is taken as the last dilution preceding complete haemagglutination.

Infectivity titrations — Tenfold serial dilutions of virus are inoculated in 0·1 ml aliquots into 5-day-old mouse embryo fibroblasts (M.E.F.) and M.K. tissue cultures. The end point is determined by the method of Reed and Meunch [5], on the basis of cytopathic changes. The material from a negative tube is passed as a further test and experiments are terminated 4 weeks after inoculation.

Plaque assays — These are performed on 5-day-old M.E.F. and M.K. primary monolayers in plastic petri dishes with an initial overlay of 8 ml 0·9 per cent agar containing TRIS buffer (final conc. 0·01M). This is followed on the

fourth day by a further 4 ml of agar with sodium bicarbonate buffer and neutral red at a final concentration of 1 : 20,000. Plaques are counted daily from the tenth day onwards.

Tumour origin — The fibrosarcoma was induced by polyoma virus in a C3H mouse. The tumour has been transplanted subcutaneously for 105 generations and has ten tissue culture infectious doses of polyoma virus in M.K. cultures [16]. It has been adapated to grow as single cells in the intraperitoneal cavity and will grow well in tissue culture; but since the peritoneal fluid is often haemorrhagic, trypsinization of solid intraperitoneal tumours at 4°C is used to obtain single cell suspensions. The viability of these cells in animals is determined by the growth of tumour with reference to the number of cells inoculated; 10^4 cells were necessary to produce a tumour in 19 days.

Transplantation for immunity tests — Appropriate cell suspensions are counted and diluted in fresh media then immediately inoculated subcutaneously in 0·2 ml volumes in the right or left lower abdominal quadrants. Tumour growth is recorded daily [6].

Cloning — Single cell suspensions, obtained by trypsinizing I.P. tumours overnight at 4°C, are serially diluted in complete medium. (10% calf serum, 1% sodium bicarbonate, 89% Eagles M.E.M. plus antibiotics) and incubated in a CO_2 incubator at 37°C.

One of the plates is examined the following day to ensure that there are no clumps of cells. Isolated colonies are selected and marked on the seventh day and permitted to grow until they contain approximately 200 cells. These are trypsinized after washing twice with P.B.S. A silicone greased glass cylinder is placed over the colony and 0·25% trypsin added for 2 min. The cell suspension is gently pipetted and transferred to a new plate containing medium with 20% calf serum. This procedure is repeated to establish a clone [7].

The clones are subcultured every 5 days. The cells are washed twice with P.B.S. and 2 ml of 0·25% trypsin is added for 1 min. This is removed and the plates incubated for 30 min in a CO_2 incubator at 37°C before suspending the cells in fresh media (10% serum).

Growth in agar — Isolated colonies grown in 0·3 % agar [8] are picked off the agar plate with a Pasteur pipette and plated in medium, where the cells grow out from the agar. This method has been used for recloning the cells.

EXPERIMENTS AND RESULTS

Infectivity titrations

The most sensitive method for the detection of polyoma infectivity was sought by comparing results obtained by the plaque technique in plates and specific cytopathic changes in tube cultures. Both methods were tested in M.K.and M.E.F. cultures.

M.K. cultures were confirmed as more sensitive to polyoma infection than M.E.F. [1]. In tube titrations M.K. cells show a 10- to 100-fold higher titre than M.E.F. Using the plaque technique the difference is 2- to 5-fold. The titre was greater in both tissue cultures systems when tested in tubes.

Polyoma growth curves show a higher virus yield in M.K. cultures (Fig. 1).

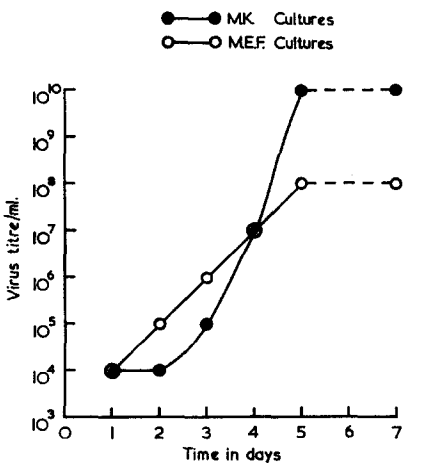


Fig. 1. Production of M.H.P. virus in mouse tissue cultures. Virus yield is greater in mouse kidney cells than in mouse embryo fibroblasts.

Inoculation of 10^6 T.C.I.D. of virus into M.K. and M.E.F. cultures produced 10^{10} T.C.I.D. and 10^8 T.C.I.D./million cells respectively. The mean production of infectious virus particles was 10^4 per M.K. cell and 10^2 T.C.I.D. per M.E.F. cell.

Infectivity was compared with the number of virus particles estimated from the haemagglutination titre. Ten virus particles constituted 1 T.C.I.D. for M.K. cells, and 10^3 for M.E.F. cultures. So for all infectivity experiments, M.K. cultures were used.

Clones

Cloning of the tumour produced two types of cells.

Clone 1 (*C.*1) — This consists of elongated fibroblastic cells often overlapping and never forming a continuous sheet (Fig. 2). C.1. has

shown stable morphology for seventy-three generations and four sub-clones have the same characteristics.

They have limited growth in tube cultures and degenerate completely after 10 days. When 10^6 C.1 cells were added to tube cultures of normal M.K. cells to form mixed cultures, no contact inhibition was seen between the two types of cells. The cloned cells grew on top of the kidney cells (Figs. 3 and 4). Passage of the medium from these mixed cultures into fresh M.K. cultures produced cytopathic changes, and this C.P.E. was neutralised by rabbit anti-polyoma antiserum. So far, this infectivity has been demonstrated 8 times; 10^7 C.1 cells are required to produce one infectious dose. This is of a comparable order with the mutagenic rate of bacteria. There is a possibility of viral contamination but passage of control culture medium has always been negative.

The C.1 cells are transplantable in C3H mice and tumours are produced in 10–12 days with 10^4 cells given subcutaneously.

Clone 2 (*C.*2) — Clone 2 consists of smaller polygonal cells growing in a close network arrangement. (Fig. 5). They can be maintained in culture for 6 weeks provided the medium is changed daily. Frequently contact inhibition was seen when 10^6 cells were grown with M.K. cultures and the C.2 cells were arrested at the edge of the epithelial sheet (Figs. 6 and 7). As yet no infectivity has been demonstrated from these cultures.

They are not transplantable in C3H mice (even after irradiation and cortisone treatment) with doses of 2×10^6 cells. Inoculation into new-born mice has proved negative. Tumours do arise at the site of inoculation at approximately the same time as C.1 cells but regress after 5–9 days.

Addition of 10^6 C.2 cells to M.K. tube cultures delayed the production of C.P.E. 5 days in polyoma virus titrations and resulted in a 10-fold drop in titre. Comparable numbers of C.1 cells and normal fibroblasts had no effect on titrations. Two morphological variants have been obtained in seven subclones from C.2, but these still retain their immunological properties and no infectivity has been demonstrated in these sub-clones. Both clones when infected with varying doses of virus, produce less virus than comparable M.E.F. cultures.

The clones can be frozen using dimethyl sulphoxide and stored at −70°C [9].

Transplantation and immunity experiments

C3H mice were inoculated subcutaneously with 10^6 C.2 cells. Any resulting growth regressed within 9 days and on the fourteenth day the mice were inoculated subcutaneously on the opposite lower abdominal quadrant with varying doses of C.1 cells. A high proportion of tumours arising from these cells regressed or failed to develop. The sera of all animals were tested for polyoma haemagglutination inhibition (H.I.). Twelve out of 151 mice gave positive H.I. results and were not included in totals of the following tables [10].

Control animals were bled at the beginning and end of each experiment; two out of thirty-six had positive inhibition.

Table 1. *Specific antigens in cells from a polyoma induced mouse fibrosarcoma*
(*C.*1 = *Malignant cells*
*C.*2 = *Non-malignant cells*)
*Transplantation of C.*1 *cells in mice*

Number of C.1 cells inoculated	Mice without prior treatment (Number of animals with tumours / Number of animals inoculated)	Mice previously inoculated with 10^6 C.2 cells (Number of animals with tumours / Number of animals inoculated)	Mice previously inoculated with 10^6 normal mouse cells (Number of animals with tumours / Number of animals inoculated)
10^6	14/16	3/10	
10^5	18/23	2/9	6/6
10^4	20/24	1/10	6/6
10^3	6/16	1/10	

From these preliminary results (Table 1) there appears to be immunity to the C.1 cells following inoculation of C.2 cells.

C3H mice inoculated with 10^6 C.2 cells in the right abdominal quadrant, had their corresponding axillary lymph nodes removed after 14 days. A suspension of 2×10^6 lymphocytes was inoculated intravenously into other C3H mice and 10 days later they were challenged with 10^5 or 10^4 C.1 cells. The results are shown in Table 2. Only two out of six mice receiving lymphocytes prior to inoculation of 10^4 or 10^5 C.1 cells showed tumours, while five out of six mice receiving C.1 cells only had tumours.

Prior inoculations of 10^6 normal mouse fibroblast cells produced no alteration in the number of mice with tumours when compared with mice receiving C.1 cells only.

Table 2. Lymphoctyes from mice receiving C.2 cells immunize other mice against C.1 cells

Number of C.1 cells inoculated	Number of lymphocytes inoculated	Number of animals inoculated	Number of tumours
10^5	0	6	5
	2×10^6	6	2
10^4	0	6	5
	2×10^6	6	2

DISCUSSION

Two cell clones differing in morphological and biological behaviour were obtained from a polyoma-induced fibrosarcoma. One clone consists of overlapping fibroblastic cells which show no contact inhibition with normal mouse kidney cells. Infectious virus is produced from 1 in 10^7 cells and the clone is transplantable in mice. The other clone, composed of polygonal cells, grows in a close network arrangement. The cells exhibit contact inhibition, and do not produce infectious virus. Although non-transplantable in C3H mice, the clone will immunize against further tumour cell inoculation and lower the titre of polyoma titrations in normal mouse kidney tube cultures. This demonstrates the possession of an antigen in common with the transplantable clone. Both clones will grow in 0·3% agar, the transplantable clone forming larger colonies than the non-transplantable clone.

The liberation of virus from the transplantable clone has only been demonstrated 8 times, in the order of 1 in 10^7 cells. This result is in apparent contrast with that of Winocour and Sachs [11].

Excluding the possibility of viral contamination, which has never occurred in any control cultures, this suggests that the polyoma DNA is integrated in the tumour cell and by simple mutation liberates demonstrable infectious virus.

No infectivity has been detected in cells of the non-transplantable clone and we assumed at first that these were stroma cells. If this were so then they were not derived from uninfected cells in the host but from the tumour since both types of cell possess a common antigen.

It is easier to assume either that each clone derives from a different cell, or that the non-transplantable line arises from the transplantable line by mutation. If this is so, then the antigen does not depend on polyoma DNA integration.

RÉSUMÉ

Deux clones de cellules (C.1 et C.2), provenant d'une tumeur produite chez la souris C3H par le virus polyome, furent établis en cultures de tissus.

Les deux clones différaient par les propriétés suivantes: C.1, transplanté chez des souris C3H adultes, y produisait une tumeur; lorsqu'il était cultivé en présence de cellules de reins de souris, il ne provoquait pas d'inhibition de contact et libérait une très petite quantité de virus.

C.2 ne produisait pas de tumeur chez la souris C3H adulte; lorsqu'il était cultivé en présence de cellules de reins de souris, il provoquait une inhibition de contact et ne libréait pas de virus.

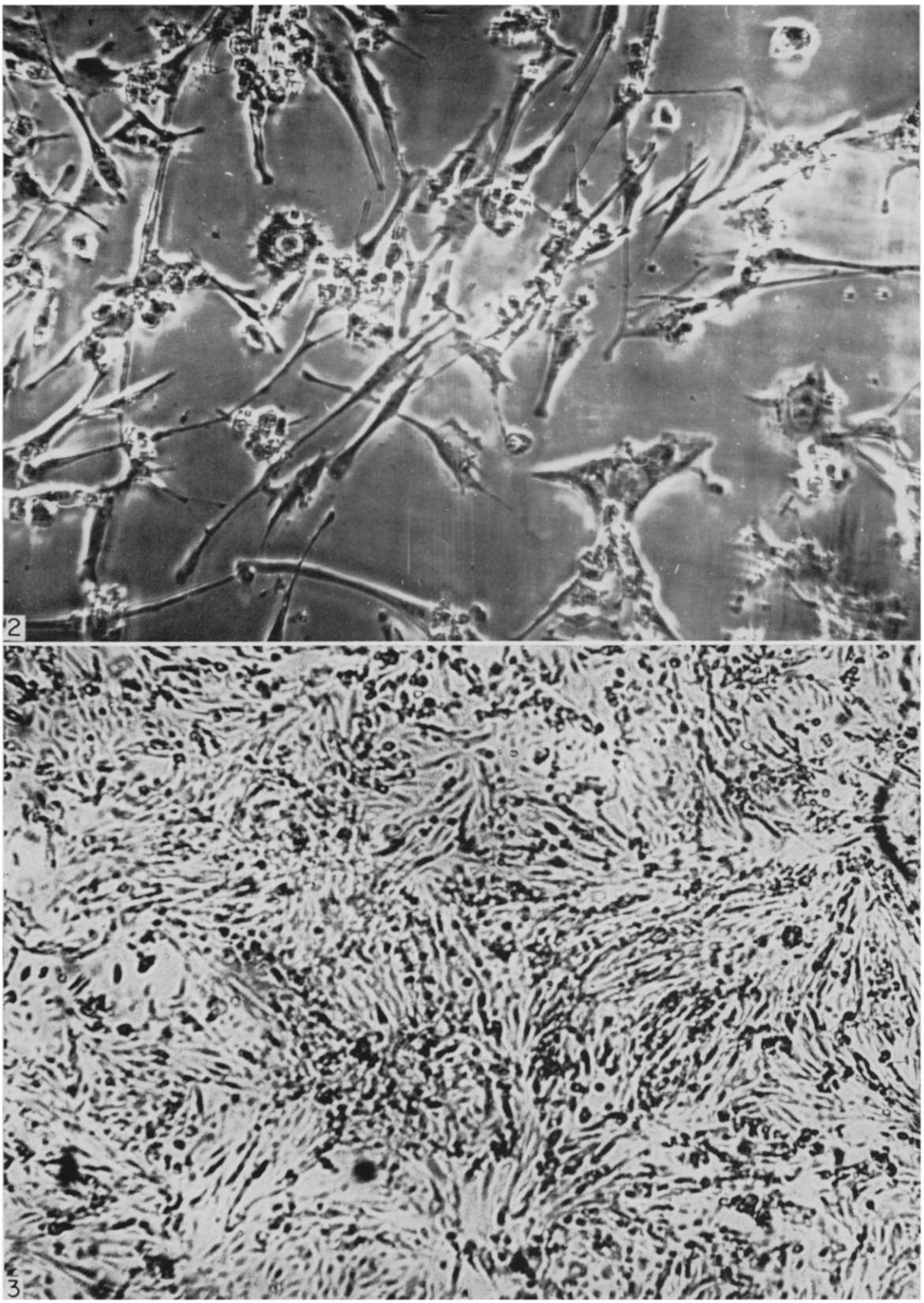
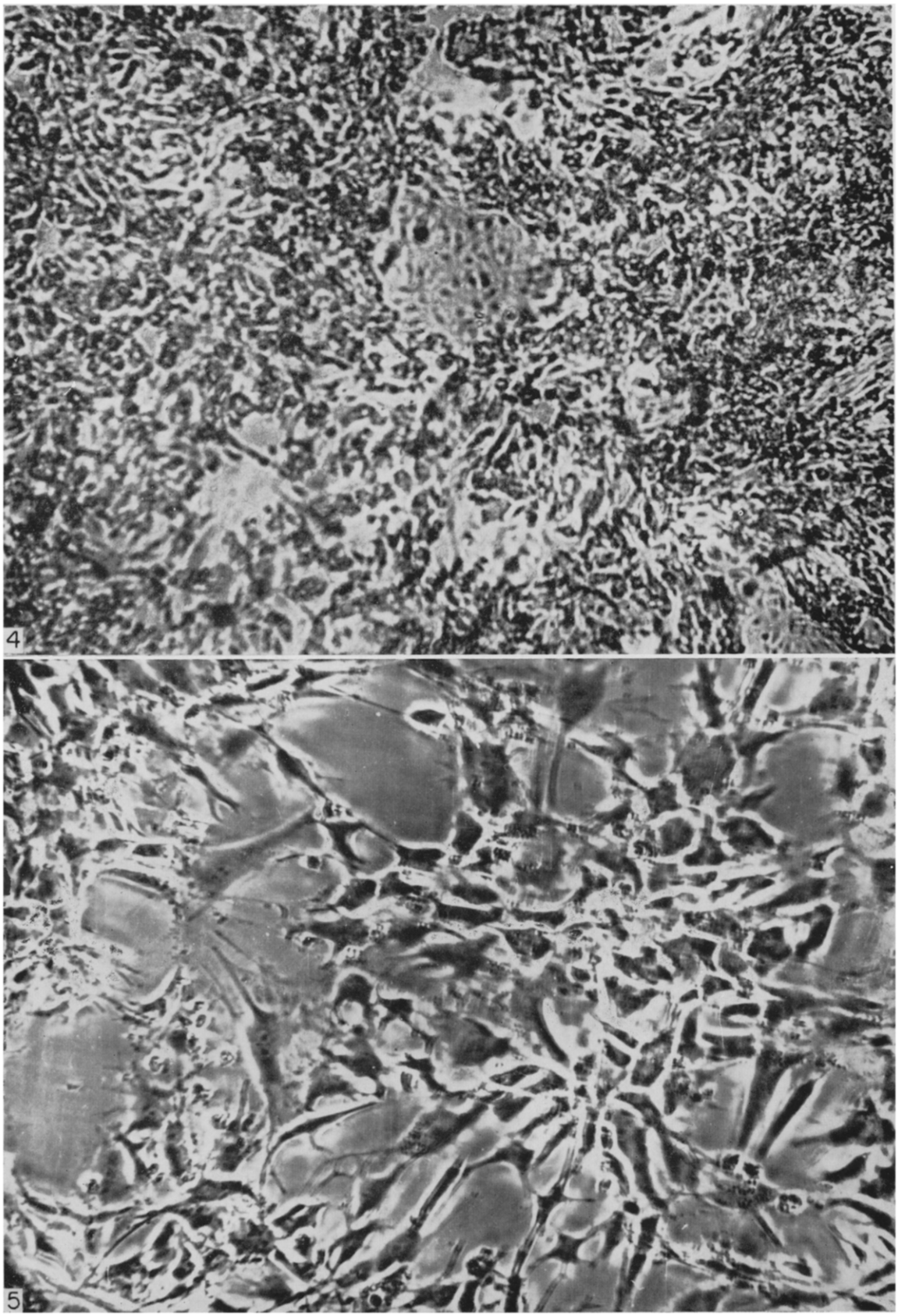


Fig. 2. Clone 1. Elongated fibroblastic cells growing on glass and overlapping. They never form a continuous sheet. × 160, 16 *mm cruciform phase contrast objective.*

(to face p. 224)



Figs. 3 *and* 4. *Clone* 1 *cells, grown with mouse kidney cells. No contact inhibition is seen between the two types of cells. The cells from Clone* 1, *which in these pictures appear dark, grow on top of the mouse kidney cells.* × 60.

Fig. 5. *Clone* 2. *Small polygonal cells growing in a close network and forming a sheet.* × 160, 16 *mm cruciform phase contrast objective.*

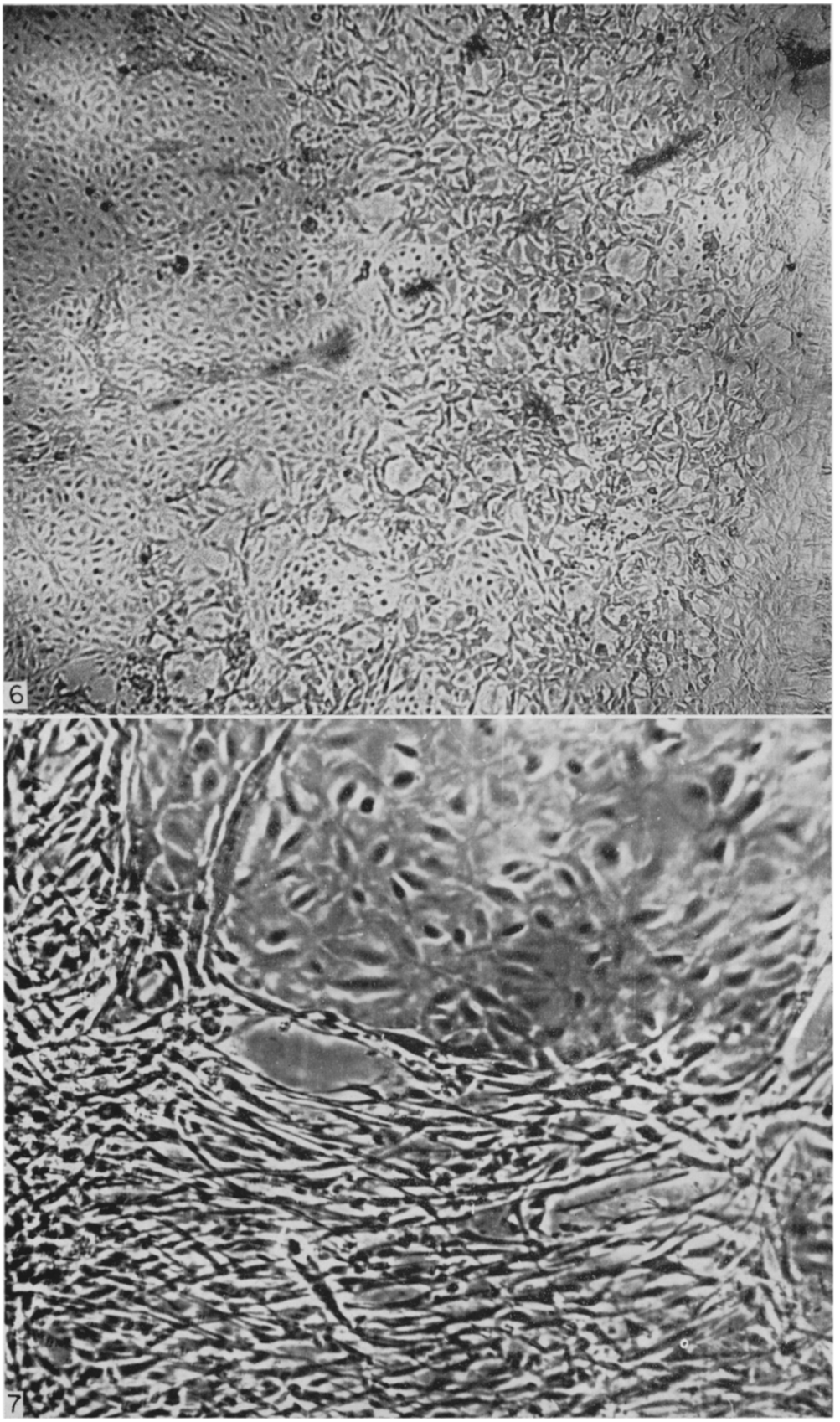


Fig. 6. *Clone* 2 *cells, on the right, mouse kidney cells in this picture, grown with mouse kidney cells seen on the left. Note the contact inhibitions. The C.*2 *cells are arrested at the edge of the epithelial sheet.* × 60.

Fig. 7. *The same as in Fig.* 6. × 160, 16 *mm cruciform phase contrast objective.*

Cependant, les cellules de C.1 et C.2 possédaient un antigène de transplatation commun. Ces faits suggèrent qu'il y a au moins deux modes d'interaction différents contre le virus polyome et la cellule murine.

SUMMARY

Two cell clones (C.1 and C.2) derived from a polyoma-induced C3H mouse tumour, were established in tissue cultures.

The two clones differed in the following properties: C.1 when transplanted into adult C3H mice, produced a tumour; when grown together with mouse kidney cells showed no contact inhibition and liberated a very small amount of virus.

C.2 produced no tumour in adult C3H mice; when grown together with mouse kidney cells showed contact inhibition and did not liberate virus.

However, the cells from C.1 and C.2 shared a common transplantation antigen. These findings suggest that there are at least two different ways of interaction between polyoma virus and mouse cell.

ZUSAMMENFASSUNG

Es wurde zwei Zellreihen (C.1 und C.2), die aus einem durch das Polyoma-Virus provozierten C3H-Mäusetumor stammen, in Gewebskulturen gezüchtet.

Die zwei Zellreihen unterschieden sich von einander in folgenden Beziehungen:

C.1 riefen in erwachsenen C3H-Mäusen Tumoren hervor; wenn sie zusammen mit Mäusenierenzellen gezüchtet wurden, ergab sich keine Kontaktinhibition und kleine Virusmengen wurden freigelassen.

C.2 produzierten keine Tumoren; gegenüber den Nierenzellen kam ez zu Kontaktinhibition und kein Virus wurde friegelassen.

Anderseits teilten sich C.1 und C.2 ein gemeinsames Transplantationsantigen.

Diese Feststellungen erlauben den Schluss, dass es zwischen dem Polyoma-Virus und Mäusezellen mindestens zwei verschiedene Interaktionengigbt.

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The Effects of Cortisol and Chlorazanil on the Mitotic Rate in Mouse Liver and Skin

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IT HAS been demonstrated [1] that the diuretic drug chlorazanil (2-amino-4(p-chloroanilino)-s-triazine) inhibits the accumulation of glycogen and of amino-acids induced in the liver by cortisol. Chlorazanil also slightly depresses the effect of androgens on muscle mass but it does not affect the action of oestrogens nor the anti-inflammatory action of cortisol.

Cortisol has been shown to have an anti-mitotic effect on the liver [2] and skin [3]. Furthermore, Bullough and Eisa [4, 5] suggested that glycogen metabolism is intimately concerned in the mitotic process. Since chlorazanil blocks cortisol-induced glycogenesis, it was decided to investigate whether the anti-mitotic action of cortisol was also inhibited by chlorazanil.

METHODS

All experiments were performed on adult albino mice. The animals used were males, in order to eliminate any possible effects of the oestrus cycle, and they were all killed at 4.0 p.m. to avoid variations due to the diurnal rhythm of mitosis that is found in skin [6] and in normal [7] and regenerating liver [8]. Unless indicated otherwise, food and water were allowed *ad libitum* throughout all the experiments. Mitotic arrest was produced by colchicine (100 μg) given by subcutaneous injection 6 hr before killing.

In the investigations on liver regeneration, partial hepatectomy was performed by the standard method [9] carried out between 2 and 4 p.m.: the mice were killed 48 hr later. The methods used for assessing regeneration by weight and by mitosis counts have been described previously [10]. For the skin experiments, skin was obtained from the interscapular region and mitosis counts were performed as in a previous study [11].

The cortisol (as hemisuccinate) was dissolved in normal saline. The chlorazanil was dissolved in saline acidified to pH 2·0 with hydrochloric acid. A few grains were left undissolved but were injected in suspension.

RESULTS

The animals were partially hepatectomized and divided into six groups.

Group I: controls subjected to partial hepatectomy only.

Group II: twenty-one mice were injected with normal saline (0·1 ml) in two doses subcutaneously and fifteen mice with normal saline (1 ml) intraperitoneally in one dose: the results were almost identical and are combined in Table 1.

Group III: acid saline (pH 2·0) was injected in doses of 0·3 ml intra-peritoneally, one injection being given immediately after the partial hepatectomy and the second dose 24 hr later (i.e. 24 hr before death).

Group IV: 0·5 mg cortisol was given in two subcutaneous doses administered at the same time as the injections in Group III.

Group V: two doses of 150 μg of chlorazanil in 0·1 ml of acid saline were injected intra-peritoneally at the times given for Group III.

Group VI: these mice received both chlorazanil and cortisol as in the schedules of Group IV and V, the two drugs being given at different sites.

Effects on skin regeneration

This series also is divided into six groups (Table 2). It will be seen that a short period of fasting produced no significant effect. On the other hand acid saline did cause a fall in the mitotic rate. Chlorazanil and cortisol administered separately were both potent inhibitors but when the two drugs were given simultaneously, the effects were quite clearly not additive.

DISCUSSION

The fact that exogenous cortisol depresses mitotic activity in the skin and the liver is well known. Endogenous cortisol, evoked by the stress of any slightly painful injection (such as, in the present case, acid saline) has been shown to lower the mitosis rate in regenerating liver [12] and this mechanism presumably also accounts for the moderate lowering of the mitotic count in the skin following the injection of acid saline

In the case of the liver, chlorazanil by itself has a marked anti-mitotic effect: this is proved by the comparison of the chlorazanil series with the acid saline controls. The mechanism of this is not known. It is possible that both cortisol and chlorazanil act on mitosis by causing a shortage of the amino-acids required for cell division. Swann [13] emphasized that the actual process of cell division requires the formation of new types of protein, and, of course, synthesis of further cell protein. Cortisol, by diverting amino-acids into glyconeogenesis, may

Table 1. *The effect of cortisol and chlorazanil on the regenerating liver of the mouse, 48 hr after partial hepatectomy*

Experimental group	No. of mice	Percentage increase in wet weight (±S.E.)	% mitotic rate (±S.E.)	Significance of mitotic rate Against	p
(1) No treatment	36	72·5±3·5	3·25±0·59	—	—
(2) Saline	36	63·3±3·8	3·06±0·37	—	—
(3) Acid saline	22	56·6±5·3	1·10±0·25	Saline	$<0·001$
(4) Cortisol	20	64·9±4·4	0·31±0·11	Saline	$<0·001$
(5) Chlorazanil	20	56·9±4·4	0·43±0·15	Acid saline	$<0·05$
(6) Chlorazanil + cortisol	14	71·2±6·1	0·04±0·02	Acid saline	$<0·005$
				Chlorazanil	$<0·05$

These findings are summarized in Table 1. The injections of normal saline produced a negligible effect on mitotic rate but the acid saline caused a significant fall. Cortisol and chlorazanil separately each produced an even greater lowering of mitotic rate, which was significant against the normal saline controls and the acid saline controls respectively. Finally, cortisol and chlorazanil together caused a profound fall; of the fourteen mice in this group, thirteen showed no mitoses, and one gave a value of 0·64 per cent.

Although the different groups showed some variation in the mean value for the increase of wet weight of the liver remnant, the variations are not statistically significant. This poor correlation between wet weight increase and mitotic count has been encountered previously [10] and it has been argued that the mitotic count is a more accurate reflection of liver regeneration [12].

Table 2. *The effect of various procedures on the mitosis rate in the epidermis*

Experimental group	No. of mice	Mitosis/cm (± S.E.)	Significance Against	p
Normal	50	31·2±3·4	—	—
Fasted for 24 hr	25	26·2±4·9	Normal	$0·4 < p < 0·5$
Acid saline controls	25	20·6±5·4	Normal	$0·05 < p < 0·1$
Chlorazanil	25	8·1±1·1	Normal	$p < 0·001$
Cortisol	25	14·7±4·3	Normal	$0·001 < p < 0·01$
Cortisol and chlorazanil	24	15·9±3·3	Normal	$0·01 < p < 0·02$
			Acid saline	$0·6 < p < 0·7$

cause a local shortage for other purposes, and, as Williamson and Shideman [1] showed, chlorazanil inhibits the accumulation in the liver of amino-acids. It has been claimed [14] that deficiency of amino acids inhibits mitosis in growing tissue. It is, therefore, possible that the two drugs together cause such a profound shortage of amino-acids that cell division virtually ceases, though it is admitted that this explanation is very tentative.

Bullough [15–18] has speculated that the cell concentration of glycogen or glucose is the most important single factor affecting mitotic activity. However, the fact that chlorazanil blocks the accumulation of glycogen in the liver caused by cortisol, while it actually enhances the anti-mitotic effects, suggests that a quite different mechanism is involved.

In the skin chlorazanil itself has a depressant effect, significant when tested against the acid saline controls. Any possible anorexia induced by the drug could not account for this action, because starvation for 24 hr does not produce a significant depression. In contrast to the liver, chlorazanil together with cortisol caused no more depression than either drug separately. These results emphasise once again that it is difficult to generalise about the anti-mitotic actions of drugs, and each tissue must be considered separately.

Despite the lack of understanding of the mechanism involved, the fact that the combination of drugs causes a very marked depression of mitotic activity in the liver and that this is, at least to some extent, selective, suggests that this combination might be of therapeutic value in hepatocellular carcinoma of the liver. Experiments to test this possibility are in progress.

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RÉSUMÉ

Le cortisol et le diurétique "chlorazanil," administrés séparément, dépriment la division cellulaire dans le foie en régénération, après hépatectomie, chez la souris. Lorsque ces substances sont administrées simultanément, elles produisent une disparition presque complète de l'activité mitotique.

Le chlorazanil et le cortisol, donnés séparément, provoquent aussi une dépression modérée de l'activité mitotique dans l'épithélium de la peau normale. Contrairement à ce qui se passe dans le foie cependant, l'action combinée des deux substances n'est pas plus grande que l'activité de chacune d'elles utilisée isolément.

SUMMARY

Both cortisol and the diuretic drug chlorazanil given separately depress cell division in the regenerating liver of the mouse following hepatectomy. When the substances are given simultaneously, an almost complete suppression of mitotic activity is produced.

In the normal skin chlorazanil and cortisol given separately again cause moderate depression of mitotic activity in the epithelium. By contrast with the liver, however, the combined action of the two drugs is no greater than that of either separately.

ZUSAMMENFASSUNG

Sowohl Cortisol als auch das Diuretikum Chlorazanil, wenn getrennt verabfolgt, setzen die Zellteilung in der regenerierenden Leber nach einer Hepatectomie bei der Maus herab. Wenn beide Substanzen gleichzeitig verabfolgt werden, kommt es zu einer fast vollkommenen Unterdrückung der Mitosenaktivität.

In der normalen Haut rufen sowohl Cortisol als auch Chlorazanil, wenn getrennt verabfolgt, eine mässige Depression der Mitosenaktivität des Epitheliums hervor. Im Gegensatz zu der Leber ist die kombinierte Aktivität beider Substanzen nicht bedeutender als die getrennte Wirkung.

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Studies on Lymphopoiesis—IV

A Comparison of Two Approaches for the Determination of the Generation Time in Thoracic Duct Cells without Detectable Cytoplasmic Differentiation*

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THE PROLIFERATIVE pattern of many human malignant neoplasias corresponds at least in part to that of a cell line without detectable cytoplasmic differentiation. Since the growth of such a cell family does not result in the production of a morphologically defined and unequivocally differentiated end cell, only a limited number of possibilities remains for analysis after labelling with tritiated thymidine.

Feinendegen [1] has recently reviewed in detail the many possible pitfalls in the interpretation of autoradiographic results. It is desirable to find a technique of evaluation that necessitates a minimum number of assumptions [2].

The purpose of this short communication is to discuss the respective validity of two methods suitable for cytokinetic studies on cell lines without detectable cytoplasmic differentiation:

1. the change of the labelling index of mitotic figures and of the mean grain count per labelled mitotic figure and
2. the decrease of the mean grain count per labelled interphase cell,

both as a function of time after a single injection of tritiated thymidine.

For this discussion, the lymphocytic cell line was chosen as a model system since its lack of cytoplasmic differentiation and possible potentiality for further proliferation bears some resemblance with certain human neoplastic diseases.

MATERIALS AND METHODS

A 4-months-old calf of approximately 200 lb received an extracorporeal irradiation (ECI) of the peripheral blood of 48 hr as described previously [3]. During ECI the dose to red cells accumulated so that it was distributed between a minimum of 66×10^3 and a maximum of 110×10^3 rads [4]. After removal of the extracorporeal loop the thoracic duct was canulated for continuous drainage and reinfusion of the lymph. A single intravenous injection of 0·2μ c/g body weight of thymidine-^{3}H (Schwartz Bio Research), specific activity: 1·9 c/mM, concentration: 0·1 mc/ml was given. Lymph samples were obtained at 10-min intervals for 3 hr, at 30 min intervals up to 10 hr and at 1-hr intervals up to 22 hr after injection of the marker. The lymph was centrifuged at room temperature (21°C, 1000 rev/min) and the supernatant reinfused. Smears were made with the sedimented cells, dried and fixed in absolute methyl alcohol for 15 min. Several

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sets of slides were processed for autoradiography with liquid emulsion (Eastman Kodak NTB-2). The set used for this study was exposed for 14 days at 4°C in dehumidified air. Other sets were used as controls. After development, smears were stained through the film with Giemsa solution, concentration 1:20, at pH 6·0, dried and mounted with coverslips.

On all slides except three, at least 100 mitotic figures were counted. Registering of early prophases as well as of late telophases was avoided. The number of grains over the nuclear material of each mitotic figure was noted. Background was corrected for by measuring the mean nuclear surface area of 100 mitotic figures and by counting the grains over 100 similar cellfree areas within the same smear.

On the same set of slides cells in interphase were classified according to nuclear size as reported earlier [5]. On each slide fifty interphase cells with nuclear sizes six and five and fifty with nuclear sizes four and three were counted. Cells with nuclear sizes two and one were not registered since they were not labelled initially, i.e. at 1 hr after injection of thymidine-^{3}H. The number of grains over the nucleus of each cell was counted. Background was corrected for as indicated above.

RESULTS

The change in the *labelling index of mitotic figures* (mitotic labelling index) as a function of time after a single injection of tritiated thymidine is shown on the upper part of Fig. 1. The first labelled mitoses appear in the sample taken 30 min after injection of thymidine-^{3}H. At 90 min, 99 per cent of the mitotic figures are labelled. The mitotic labelling index curve then remains very close to 100 per cent until 4 hr. Thereafter, a significant decrease over a 90-min period is observed. This variation of the mitotic labelling index curve is interpreted as suggestive of a short DNA synthesis time.

The change of the *mean grain count per labelled mitotic figure* as a function of time after a single injection of thymidine-^{3}H is represented on the lower part of Fig. 1. The curve demonstrates two peaks separated by an interval of approximately 5½ hr. The second peak is probably due

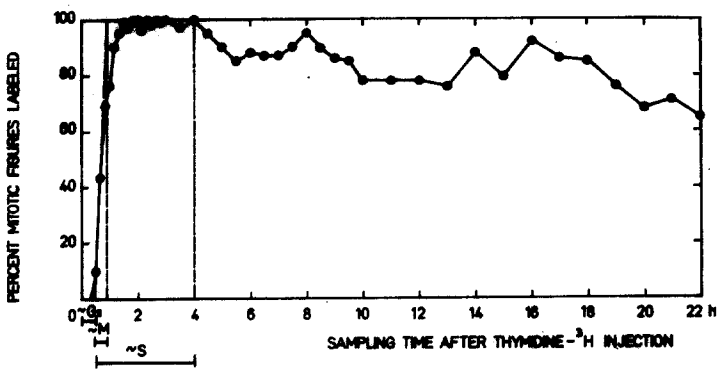
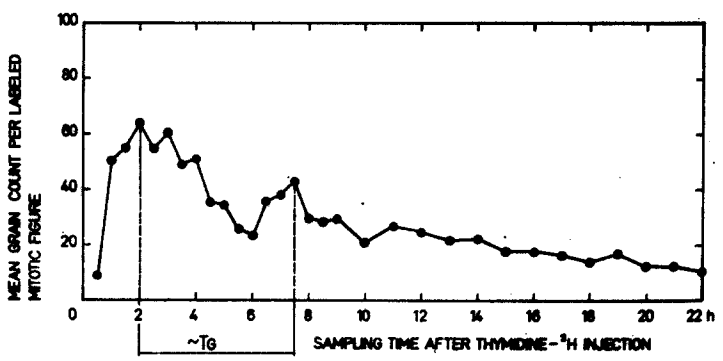


Fig. 1a. Mitotic labelling index curve for all mitotic figures in calf thoracic duct after extracorporeal irradiation of the blood and after one thymidine-^{3}H injection.



b. Mean grain count per labelled mitotic figure in calf thoracic duct after extracorporeal irradiation of the blood and after one thymidine-^{3}H injection.

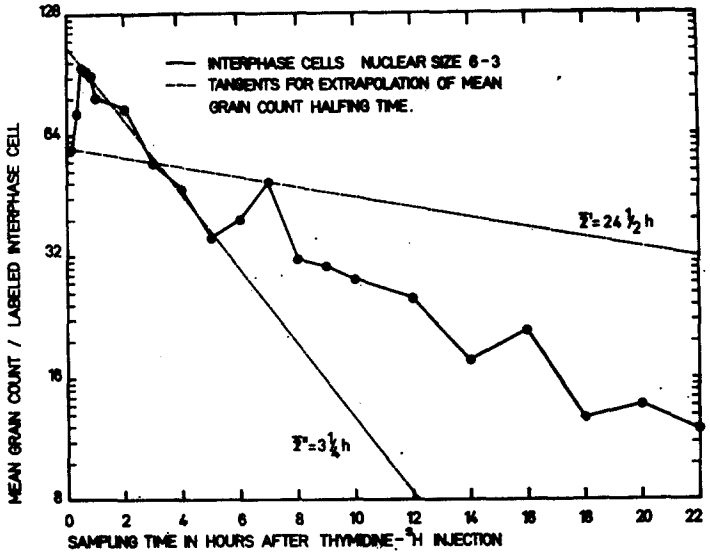


Fig. 2. Interphase cells in calf thoracic duct after extracorporeal irradiation of the blood and after a single intravenous injection of thymidine-³H.

to cells undergoing a second cycle after injection of tritiated thymidine. Practically identical results were obtained on two other calves.

A generation time of 5½–6 hr for the majority of cells becomes even more evident. when less basophilic mitotic figures are excluded from the counts [6]. No difference is observed when large mitotic figures are compared with medium sized ones.

The change of the *mean grain count per labelled interphase cell* with nuclear sizes six to three as a function of time after a single injection of thymidine-³H is shown on Fig. 2. The mean grain count first rises up to 30 min for all cells counted. This is probably due to prolonged incorporation of the marker into macromolecular DNA. Then it starts to drop abruptly when the first labelled mitotic figures appear. Two peculiarities become apparent in the further shape of the curve:

1. the slope is not constant on a semilogarithmic plot and
2. at least one peak appears around 7 hr after thymidine-³H injection.

Separate mean grain count curves for labelled interphase cells with large and medium sized nuclei are shown on Fig. 3. In general the overall mean grain count per labelled cell is lower for lymphoid elements with medium size nuclei than for those with larger nuclei.

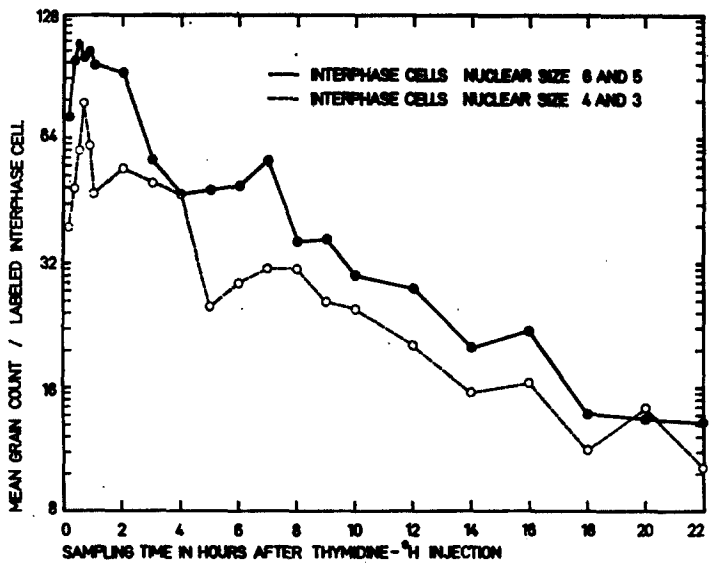


Fig. 3. Interphase cells in calf thoracic duct after extracorporeal irradiation of the blood and after a single intravenous injection of thymidine-³H.

DISCUSSION

The combined analysis of the change of the mitotic labelling index and of the mean grain count per labelled mitotic figure as a function of time after a single injection of thymidine-^3H permits a rather precise determination of the generation time and of the time parameters of the different phases of the generation cycle. This holds even for a cell family without detectable cytoplasmic maturation, provided that cells with varying nuclear size — as shown in this experiment — do not differ in their proliferation characteristics.

The main disadvantages of this method are:

1. the necessity for frequent and prolonged sampling which might restrict its feasibility or which might interfere with existing steady states,
2. the difficulty of reconizing mitotic figures as belonging to a given cell type in interphase.

These disadvantages led to a search for less demanding methods, such as the assessment of the *mean grain count halving time for initially labelled cells in interphase* as a first estimate of generation time. In order to determine accurately a mean grain count halving time, one should obtain:

1. the initial mean grain count per labelled cell in interphase and
2. a sufficient number of mean grain counts per labelled interphase cell at later time intervals which may give a straight line at least over a certain period of time on semi-logarithmic plot (such as Figs. 2 and 3).

The following factors may hinder the evaluation of generation times on the basis of the mean grain count halving time for labelled cells in interphase:

1. In a cell line with a short duration of G_2 (premitotic gap) and M (mitosis), such as the one examined here, the mean grain count per labelled interphase cell as a function of time is determined initially by at least two different processes: the prolonged incorporation of the cell marker into macromolecular DNA and the first divisions of labelled cells. If the mean grain count does not level off before the drop due to first divisions occurs, a precise determination of the initial mean grain count is difficult or impossible to obtain.
2. As shown in the lower part of Fig. 1, labelling with tritiated thymidine is most intense in cells that are in the second half of DNA-synthesis phase during availability of the precursor. Therefore, the majority of the more heavily labelled cells are expected to divide earlier than less heavily labelled ones. This will cause a steep initial drop in the mean grain count curve of cells in interphase. Such a drop could very well simulate too short a generation time if the estimate of the latter is based on mean grain count halving time.
3. If initially labelled cells divide only once after thymidine-^{3}H injection and then stop giving further birth to cells without changes in morphology, the mean grain count will remain too high, the slope too flat and the estimated generation time too long.
4. If labelled nuclear debris appear rapidly after thymidine-^{3}H injection — as they actually do in lymphatic tissue — early re-utilization of thymidine-^{3}H may occur resulting in weak labelling of initially unlabelled but proliferating cells. This, of course, would depress the mean grain count, make the slope of the mean grain count curve too steep and the estimated generation time too short.
5. In determining the mean grain count halving time, only such cell types should be considered that have a high initial labelling index, e.g. that are continuously proliferating. Therefore cell types corresponding to initially unlabelled cells have to be excluded from these counts. If this is done, an initially labelled cell will not be registered any more as soon as it develops into a cell with morphologic characteristics corresponding to those of an initially non-labelling cell-type. In the calf thoracic duct, lymphoid cells with nuclear sizes six and five have a higher initial mean grain count than cells with medium sized nuclei (Fig. 3). Cells with nuclear sizes two and one have not been registered since they are initially unlabelled, but it was found that an appreciable number of these cells were labelled after 2–4 hr. It is not unlikely that they develop from cells with nuclear size three. If, therefore, at approximately 6 hr an increased number of initially labelling cells of medium nuclear sizes will have developed into cells with small nuclei, the mean grain count of labelled cells with nuclear sizes six to three will show a relative increase, as seen on Figs. 2 and 3.

When determining the slope of the mean grain count curve of cells in interphase in order to define the mean grain count halving time on the basis of the values of Fig. 2 and without considering the factors listed above, it becomes highly critical at exactly what time intervals after thymidine-^{3}H injection, the first as well as the subsequent mean grain counts are obtained. As shown on Fig. 2, such widely different grain

count halving times as 3½ and 24½ hr may be obtained by chance alone. We know from the mitotic labelling index data that both these values do not correspond to the actual best estimate for the majority of cells, that is 5½ hr for generation time. In addition it is quite obvious that the slope representing a mean grain count halving time of 5½ hr could not be covered satisfactorily by subsequent mean grain counts on Fig. 2.

These results indicate that methods using the mean grain count halving time as an estimate of generation time are less reliable than those based on mitotic labelling indices in combination with data on mean grain count per labelled mitotic figure.

For *in vivo* cytokinetic studies on human neoplastic cells, clinical situations should be sought, in which material with sufficient numbers of mitotic figures can be obtained at frequent intervals over a prolonged period of time and without disturbing the steady state. There is a great need for such data in order to know whether it might be possible to increase the efficiency of cancer chemotherapy by adjusting the dose intervals of cytolytic or cytostatic agents to the time parameters of the generation cycle of malignant cell populations.

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RÉSUMÉ

L'étude cytokinétique in vivo *des cellules lymphoïdes du canal thoracique du veau sert de modèle à une discussion de la valeur respective de deux méthodes d'analyse applicables à l'étude de lignées cellulaires sans différenciation cytoplasmique apparente. Les résultats obtenus démontrent qu'une analyse combinée de la variation du nombre relatif de mitoses marquées et de la variation du nombre moyen de grains par mitose marquée, en fonction du temps après une seule injection de thymidine-^3H, permet une détermination plus précise de la durée des différentes phases du cycle cellulaire que l'analyse de la diminution du nombre moyen de grains par cellule interphasique marquée, et donc de la détermination de la demi-vie du nombre moyen de grains. Dans l'investigation cytokinétique* in vivo *portant sur les lignées cellulaires malignes de l'homme, il est nécessaire d'avoir affaire à des situations cliniques telles qu'elles permettent une évaluation de la variation du nombre relatif de mitoses marquées après une injection de thymidine-^3H sans pour autant perturber l'équilibre prolifératif cellulaire.*

SUMMARY

In vivo *cytokinetic studies on calf thoracic duct lymphoid cells using tritiated thymidine as a cell marker served as a model for the discussion of the relative validity of two methods of investigation of cell lines without detectable cytoplasmic differentiation. The combined analysis of the change of the mitotic labelling index and of the mean grain count per labelled mitotic figure as a function of time after a single injection of thymidine-^3H gave more reliable data on the generation cycle than the analysis of the decrease of the mean grain count per labelled cell in interphase and the determination of the mean grain count halving time. For* in vivo *studies of the proliferative pattern of human malignant cell lines, clinical situations should be sought, in which evaluation of the mitotic labelling index as a function of time after a single administration of thymidine-^3H is feasible without disturbing the steady state.*

ZUSAMMENFASSUNG

Zytokinetische in vivo- *Untersuchungen mit tritiiertem Thymidin an lymphoiden Zellen des Ductus thoracicus des Kalbes dienten als Modell für eine Diskussion der Zuverlässigkeit zweier Methoden, welche für zellkinetische Untersuchungen an Zellreihen ohne morphologisch fassbare Differenzierung des Zytoplasmas als geeignet erscheinen. Gestützt auf dieselben Autoradiogramme, ergibt die kombinierte Analyse der Variation des Mitosemarkierungsindexes und der mittleren Körnerzahl pro markierte Mitose als Funktion der Zeit nach einmaliger Thymidin-^3H Injektion genauere Daten über die zeitlichen Verhält-*

nisse der einzelnen Generationszyklusphasen als die Analyse des Abfalls der mittleren Körnerzahl pro markierte Interphasenzelle und die Bestimmung der Halbwertszeit der mittleren Körnerzahl. Für zytokinetische in vivo *Untersuchungen an malignen Zellreihen des Menschen erscheinen deshalb vor allem solche klinische Situationen in Frage zu kommen, die eine Bestimmung des Mitosenmarkierungsindexes als Funktion der Zeit nach einmaliger Thymidin-^3H Injektion ohne nennenswerte Störung des Proliferationsgleichgewichtes erlauben.*

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Effect of Radioprotectors on Damage Induced by Alkylating Agents on Tumoural and Normal Tissues*

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THE therapeutic use of antitumoural drugs is limited by the toxic effects induced by these agents on normal proliferating tissues such as bone marrow and gastrointestinal mucosa. Several attempts have been made to reduce this toxicity. For instance radioprotector drugs have been combined with alkylating agents because of the assumption that alkylating agents have a radiomimetic effect. As a matter of fact it has been established that "thiol" derivatives prevent the toxicity of nitrogen mustard *in vitro* and *in vivo* in several animal species including man, although the mechanism of this antitoxic effect is not entirely clear [1–7].

Also biogenic amines, antiserotonin agents [8–11] and psychotropic drugs such as reserpine, chlorpromazine and pentobarbital [12, 13] were reported to be effective in counteracting the lethal or the leukopenic effect of alkylating agents.

Several authors reported however that all radioprotectors are not equally effective in reducing the toxicity of different alkylating agents. For instance thiol derivatives and particularly cysteine reduce the toxicity of Merophan [3, 14] and of L-Sarcolysin [15] but are without effect on Myleran [14, 16], TEM [16, 17] or Cytoxan [15, 18–20].

The usefulness of a combination of radioprotectors with antitumoural drugs should express itself by the disappearance of toxic effects on normal tissues without impairment of the therapeutic effectiveness of the antitumoural drugs on tumour growth. It is obvious that there would be no usefulness in combining radioprotectors with radiomimetic agents if by doing so, tumour growth as well as normal tissues were protected from the toxic effects exerted by alkylating agents.

The studies carried out in tumour bearing animals are very limited and only qualitative. The inhibition exerted by 5 BAU [5-(2'-bromoacetamido)-Uracil] on the Ehrlich ascites carcinoma was found to be prevented by the simultaneous administration of cysteine [21]. L-cysteine but not 2-aminoethyliso-thiouronium bromide, bromhydrate (AET) was effective also in antagonizing the antitumoural action of RC-4 {phosphinic acid, bis (1-aziridinyl)-*p*,-phenylene ester) [22] but not that of A-16 [phosphinic amide, p,p-bis (1-aziridinyl)-N] benzoyl-} [23].

MAB (1-mercapto-2-aminobutane) and cysteamine were found to prolong the survival of L 1210 leukaemic mice treated with nitrogen mustard but not to affect the survival of Gardner lymphosarcoma bearing mice in similar conditions [20].

The antitumoural effect of cytoxan, of chloroquine-mustard [20] and of L-sarcolysin [24, 25] was not modified by cysteamine or MAB. Cysteamine reduced the mortality of Walker 256 tumour bearing rats by 50% when given before a lethal dose of nitrogen mustard [26].

The protection exerted by AET on the toxicity of nitrogen mustard was found to vary with the different tissues, the cancer cells being less sensitive than the normal cells [27, 28]. Some authors [29, 30] but not others [31] found

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that AET concentrates more in normal than in neoplastic tissues.

Finally it was reported that the toxicity of some alkylating agents may be increased by radioprotectors in tumour bearing animals [32, 33].

This paper reports a systematic and quantitative study concerning the interaction of several radioprotectors with different antitumoural agents in two experimental tumours, namely sarcoma 180 and Ehrlich carcinoma transplanted in mice in the solid form.

MATERIALS AND METHODS

Drugs — The following drugs were selected as radioprotectors: serotonin creatinine sulphate (5HT) (Vister, Casatenovo), L-cysteine (Carlo Erba, Milano), cysteamine-HCl (Bracco, Milano), thiourea (Carlo Erba, Milano), 2-aminoethyl-isothiouronium bromide, bromhydrate (AET) (Light Co., Colnbrook, England), 2-methyl-piperazine-dithioformic acid (DTP) (Farmacosmici, Milano) [34], morpholine salt of morpholino-dithioformic acid (FR9D) (Farmacosmici, Milano). When possible the doses of the radioprotectors were raised up to a maximum of 1 g/kg. The following antitumoural drugs were used: DL-sarcolysin; s-Triazine, 2-4-6-tris(1-aziridinyl)-(TEM); diethylamine, 2,2'-dichloro-N-methyl. HCl (HN_2); Glycine, N,N-bis(2-chloroethyl). HCl (NSC 17661); Alanine,N,N-bis(2-chloroethyl). HCl (NSC 17663); Uracil-5 fluoro- (5FU); Mitomycin C, obtained from CCNSC, Bethesda Md.; Vinkaleucoblastine sulfate (V.L.B.) (Lilly, Indianapolis, U.S.A.), cyclophosphamide (Asta Werke, Germany).

The doses used always amounted to $\frac{1}{2}$ or $\frac{1}{3}$ of the LD_{50} determined after 6 days of treatment.

Animals — Female Swiss mice from ALAL Co Milan, were used in all the experiments. In each experiment a minimum of ten animals per group was used. Animals were kept at a room temperature of 22°C (relative humidity 60 per cent) in cages of makrolon (20 × 26 × 13 cm). They were housed in numbers of ten per cage and received food (diet ALAL 56) and water *ad libitum*.

Methods

Tumour bearing animals — Sarcoma 180 was implanted subcutaneously in the interscapular area in fragments of an average weight of 10 mg. In other experiments the tumour was transplanted subcutaneously using the ascites form (about 5 million cells/mouse) diluted to 0·2 ml with phosphate buffer solution at pH 7.

Ehrlich carcinoma was also transplanted in the same area using 0·2 ml (about 5 million cells/mouse) of the ascites form.

Animals were treated daily from the sixth day and for 6 consecutive days after transplantation of the tumour. In other experiments, the treatment was given for 1 or 2 days only.

Animals were killed 13 days after transplantation. The protector was given 10 min (intraperitoneally) or 1 hr (per os) before the antitumoural drug was administered. The following determinations were carried out 7 days after the beginning of the treatment:

(a) body weight
(b) tumour weight
(c) spleen weight
(d) leucocyte counts. carried out automatically using a Coulter Counter, mod. D.

Statistical analysis — Standard error of the mean was calculated by the usual method. The *p* value was determined according to the "rank method" [35].

Table 1. *Values in control mice bearing tumours*

	Unit of measure	Average	Max. S.E.*	Min. S.E.*	No. of determinations
Ehrlich carcinoma	mg	827	240	45	320
Spleen weight	mg/100 g b. w.	980	208	41	320
Leucocytes	thousand/mm³	13·1	2·37	0·41	320
Sarcoma 180	mg	1820	462	89	150
Spleen weight	mg/100 g b.w.	1170	189	44	150
Leucocytes	thousand/mm³	21·5	7·6	0·6	150

*Always referred to groups of ten animals.
S.E. = Standard Error.

RESULTS

Table 1 shows the values obtained in control animals bearing Ehrlich carcinoma or sarcoma 180. The average, maximum and minimum standard errors, calculated in the various control group, are reported for each determination. The standard error is always calculated for a whole group of ten animals.

The most significant data obtained with selected radioprotective agents are found in Tables 2–4.

1. *Effect of cysteine* — Cysteine protected Ehrlich carcinoma from the antitumoural effects of DL-sarcolysin, cyclophosphamide, NSC 17661 and NSC 17663. The protective effect was greater in tumour than in normal tissues (leucocyte counts, spleen weight) when the treatment was given for 6 days.

Similar results were obtained in animals bearing sarcoma 180. When the treatments lasted only 1 or 2 days, the results were qualitatively similar (Table 2).

Cysteine had no protective effect if administered orally. Besides it was ineffective in protecting against HN_2, 5 FU or VLB toxicity and, in addition, it increased the toxicity of mitomycin C in mice bearing Ehrlich carcinoma (data not reported in detail).

2. *Effect of thiourea* — In the presence of thiourea there was a reduction of the effects of DL-sarcolysin in animals bearing Ehrlich carcinoma. Similarly a protection from the toxic effect of cyclophosphamide was noted. The protection generally was of the same degree for both normal and tumour tissues. However, in mice bearing sarcoma 180 there was a better protection of the spleen particularly evident against cyclophosphamide and NSC 17663 (Table 4: exp. 3, 6, 11 and 15). Thiourea was always ineffective against HN_2, Myleran and NSC 17661.

3. *Effect of cysteamine* — Cysteamine did not show any clear activity even at very high doses (200–400 mg/kg i.p. or oral). An effect on tumour growth was shown for treatments lasting only 2 days with DL-sarcolysin (see Table 3, exp. 9).

4. *Effect of AET* — AET did not show any clear effect at doses of 200 and 400 mg/kg i.p. or oral. Some effects on tumour and leucocytes but not on spleen, were apparent when treatment with NSC 17663 was given for 6 days (see Table 3, exp. 10).

5. *Effect of other drugs* — DTP and FR9D (1g/kg oral) were ineffective although they prevented in some cases the effect of cyclophosphamide or DL-sarcolysin only on tumoural growth (see Tables 3, exp. 11 and 16).

6. *Effect of 5-hydroxytryptamine (5HT)* — 5HT (125–250 mg/kg i.p. or oral) did not protect either Ehrlich carcinoma or sarcoma 180 from the effect of DL-sarcolysin and cyclophosphamide. In many instances there was an increase of the antitumoural effect of alkylating agents. Together with this effect, however, an increase of the effect of alkylating agents on normal tissues was seen (see Table 3, exp. 5, 7 and 8). This effect is not specific for alkylating agents since the toxicity of other antitumoural drugs, like mitomycin C and 5-fluorouracil, was increased as well by a previous treatment with 5HT (see Tables 3, exp. 3 and 4) [33].

CONCLUSIONS

The most interesting but not unexpected results of this investigation consist in the finding that radioprotective drugs show a different degree of protection on antitumoural drugs and on irradiation. In fact cysteine is more effective in protecting from alkylating agents than cysteamine and AET, which are both well known as powerful radioprotector agents. AET on the other hand is almost ineffective on alkylating agents. Connors *et al.* reported similar findings by using doses of both radioprotectors and alkylating agents and measuring as an end point the modification of the LD_{50} of the alkylating agents in intact rats [3, 14].

In this investigation when the treatments were repeated for 6 days there was a better protection of the tumour than of normal tissues in the same animal. During the short term treatments (1 or 2 days), there was an equal protection or even in some cases, a slightly preferential protection on normal tissues. The radioprotective drugs did not demonstrate any selectivity in respect to a given tissue except for thiourea which showed a preferential protection on spleen.

If the results of the present investigation can be transferred to therapeutic medicine, it should be concluded that the indiscriminate use of radioprotectors in combination with alkylating agents and, possibly, with irradiation, is probably not useful but even disadvantageous to achieve a better antitumoural effect.

Table 2. Combination of cysteine and alkylating agents in tumour bearing mice

Exp. No.	Pretreatment (mg/kg, i.p.)	Antitumoural drug (mg/kg, i.p.)	No. of treatments	Days between last treatment and sacrifice	Tumour	T	L	Sp
1	—	Cyclophosphamide 125	6	1	E	27±5 ‖	29±4	16±2
	Cysteine 500	,,	6	1	E	57±10	39±4	20±1
2	—	Cyclophosphamide 125	6	1	E	19±3 *	26±1 *	12±1 *
	Cysteine 1000	,,	6	1	E	67±6	44±4	21±1
3	—	Cyclophosphamide 125	2	5	E	29±3 †	77±7	55±7
	Cysteine 1000	,,	2	5	E	46±6	103±10	63±4
4	—	Cyclophosphamide 125	1	6	E	40±4 *	145±10	147±6
	Cysteine 1000	,,	1	6	E	79±12	125±10	130±8
5	—	DL-sarcolysin 6·3	6	1	E	76±16	31±4 *	19±3 *
	Cysteine 1000	,,	6	1	E	85±8	54±5	35±2
6	—	NSC 17661 4·4	6	1	E	69±9	98±7	65±4 *
	Cysteine 1000	,,	6	1	E	86±9	99±8	94±10
7	—	NSC 17663 4·8	6	1	E	55±9	82±9	59±5 *
	Cysteine 1000	,,	6	1	E	75±12	113±7	96±6
8	—	DL-sarcolysin 4·2	6	1	●S	67±19	19±2 ‡	30±3 †
	Cysteine 500	,,	6	1	●S	106±26	37±6	52±7
9	—	DL-sarcolysin 6·3	6	1	●S	48±12	16±1 *	14±1 *
	Cysteine 500	,,	6	1	●S	112±32	30±4	32±2
10	—	DL-sarcolysin 6·3	6	1	S	59±8	40±5 §	21±2 *
	Cysteine 1000	,,	6	1	S	87±14	56±7	41±3
11	—	Cyclophosphamide 125	6	1	S	19±2 *	5±1 *	12±1 *
	Cysteine 1000	,,	6	1	S	57±4	28±4	38±4
12	—	Cyclophosphamide 125	2	5	S	15±2 *	41±7 ‖	65±6
	Cysteine 1000	,,	2	5	S	45±7	91±18	83±12
13	—	Cyclophosphamide 125	2	5	S	26±2	36±6	51±6 *
	Cysteine 500	,,	2	5	S	34±6	74±15	93±5
14	—	Cyclophosphamide 125	1	6	S	52±6 ‡	151±16	124±8 †
	Cysteine 1000	,,	1	6	S	90±13	128±11	86±4

● Transplanted from solid form.

T=Tumour weight.

L=Leucocytes count.

Sp=Spleen weight.

E=Ehrlich carcinoma (solid).

S=Sarcoma 180 (solid).

Tx=toxic, mortality 10/10 mice

▲=mortality 9/10 mice.

■=mortality 7/10 mice.

▼=mortality 4/10 mice.

The values are expressed as percent (± S.E.) of the values obtained in untreated animals.

*$p<0·01$.

†$p<0·02$.

‡$p<0·05$.

§$p=0·05$.

‖$p=0·02$.

¶$p=0·01$.

Table 3. Combination of 5HT or cysteamine or AET or DTP or FR9D and antitumoural drugs in bearing tumour mice

(For the explanation of footnote symbols see Table 2)

Exp. No.	Pretreatment (mg/kg)	Antitumoural drug (mg/kg, i.p.)	No. of treatments	Days between last treatment and sacrifice	Tumour	T	L	Sp
1	—	DL-sarcolysin 6·3	6	1	E	54±6	34±2	29±3
	5HT 250 i.p.	,,	6	1	E	21±6	12±1	13±2■
2	—	TEM 1·005	6	1	E	59±5	20±4	14±1
	5HT 250 i.p.	,,	6	1	E	Tx	Tx	Tx
3	—	5-FU 29	6	1	E	51±6 *	51±4 *	51±3 *
	5HT 250 i.p.	,,	6	1	E	25±3	27±2	34±3
4	—	Mitomycin C 2·65	6	1	E	52±5	49±6	20±2
	5HT 250 i.p.	,,	6	1	E	9	20	8▲
5	—	HN_2 0·25	6	1	E	107±17 *	87±9	100±10 *
	5HT 125 i.p.	,,	6	1	E	62±4	88±7	60±4
6	—	DL-sarcolysin 6·3	6	1	●S	48±12	16±1	14±1
	5HT 500 i.p.	,,	6	1	●S	21±5	8±1	10±1▼
7	—	DL-sarcolysin 4·2	6	1	●S	67±19 †	19±2 *	30±3 *
	5HT 500 i.p.	,,	6	1	●S	15±4	7±1	13±1
8	—	Cyclophosphamide 125	2	5	S	26±2 ‡	36±6 *	51±6 *
	5HT 125 i.p.	,,	2	5	S	17±2	14±1	18±1
9	—	DL-sarcolysin 6·3	2	5	E	48±6 ¶	73±9	54±5
	Cysteamine 400 os	,,	2	5	E	83±12	86±10	65±3
10	—	NSC 17663 4·8	6	1	E	87±11	67±4 *	65±2
	AET 200 i.p.	,,	6	1	E	100±18	100±4	61±3
11	—	DL-sarcolysin 6·3	6	1	E	51±8 ‡	20±2	18±2
	DTP 1000 os	,,	6	1	E	81±11	27±5	22±2
12	—	Cyclophosphamide 125	1	6	E	36±3 ‡	93±6	102±10
	DTP 1000 os	,,	1	6	E	57±10	93±12	106±9
13	—	DL-sarcolysin 6·3	6	1	●S	26±10 †	20±3	19±2 †
	DTP 1000 os	,,	6	1	●S	73±17	36±8	40±3
14	—	DL-sarcolysin 6·3	1	6	S	54±8 ‡	88±5	85±5
	DTP 1000 os	,,	1	6	S	90±17	87±9	94±10
15	—	DL-sarcolysin 6·3	6	1	E	51±8 †	20±2	18±2
	FR9D 1000 os	,,	6	1	E	80±8	23±2	24±1
16	—	DL-sarcolysin 6·3	6	1	●S	26±10 †	20±3	19±2 *
	FR9D 1000 os	,,	6	1	●S	93±30	28±3	39±4

Table 4. Combination of thiourea and alkylating agents in bearing tumour mice.

(For the explanation of footnote symbols see Table 2)

Exp. No.	Pretreatment (mg/kg)	Antitumoural drug (mg/kg, i.p.)	No. of treatments	Days between last treatment and sacrifice	Tumour	T	L	Sp
1	—	DL-sarcolysin 6·3	6	1	E	28±7 *	16±2 *	18±3 *
	Thiourea 1000 os	,,	6	1	E	78±7	53±8	38±4
2	—	Cyclophosphamide 125	6	1	E	16±2 *	18±2	17±1
	Thiourea 1000 i.p.	,,	6	1	E	28±3	25±2	21±1
3	—	Cyclophosphamide 125	2	5	E	29±3 *	77±7 *	55±7 *
	Thiourea 1000 os	,,	2	5	E	50±6	140±19	101±12
4	—	Cyclophosphamide 125	1	6	E	40±5	117±12	111±10
	Thiourea 1000 i.p.	,,	1	6	E	60±12	112±14	118±11
5	—	Cyclophosphamide 125	1	6	E	36±3	93±6	102±10
	Thiourea 1000 os	,,	1	6	E	52±9	123±22	127±11
6	—	NSC 17663 4·8	6	1	E	87±11 §	67±4 *	65±2 *
	Thiourea 1000 i.p.	,,	6	1	E	130±16	117±10	90±5
7	—	DL-sarcolysin 6·3	6	1	●S	48±12	16±1	14±1 *
	Thiourea 500 i.p.	,,	6	1	●S	126±48	21±2	27±2
8	—	DL-sarcolysin 6·3	6	1	S	13±1 *	13±1 *	15±1 *
	Thiourea 1000 i.p.	,,	6	1	S	44±5	34±3	35±4
9	—	DL-sarcolysin 4·2	6	1	●S	67±19	19±2 ‡	30±3
	Thiourea 500 i.p.	,,	6	1	●S	90±20	30±3	43±6
10	—	DL-sarcolysin 6·3	1	6	S	54±8 *	88±5	85±5 *
	Thiourea 1000 i.p.	,,	1	6	S	127±25	98±6	125±10
11	—	Cyclophosphamide 125	2	5	S	26±2	36±6	51±6 ‡
	Thiourea 500 i.p.	,,	2	5	S	25±5	38±6	84±12
12	—	Cyclophosphamide 125	2	5	S	15±2	41±7	65±6
	Thiourea 1000 i.p.	,,	2	5	S	16±2	42±4	98±11
13	—	Cyclophosphamide 125	6	1	S	11±1	10±1	19±2 ‖
	Thiourea 1000 i.p.	,,	6	1	S	15±3	18±4	31±5
14	—	Cyclophosphamide 125	1	6	S	46±9 ‡	93±11	130±12
	Thiourea 1000 os	,,	1	6	S	70±6	115±9	159±11
15	—	NSC 17663 4·8	6	1	S	68±7 ‖	69±6 *	58±2 *
	Thiourea 1000 i.p.	,,	6	1	S	104±13	127±8	114±8

RÉSUMÉ

Une investigation quantitative a été effectuée pour établir l'utilité des radioprotecteurs associés avec des agents alkylants dans la chimiothérapie du carcinome d'Ehrlich et du Sarcome 180 *de la souris. On a mesuré le poids de la tumeur, celiu de la rate, et on a compté le nombre de globules blancs du sang.*

Les résultats montrent que les radioprotecteurs capables de protéger la rate et les globules blancs du sang, contre les effets toxiques des agents alkylants, empêchent également l'activité antitumorale de ces agents.

Même avec un grand nombre de doses, et en changeant le mode d'administration, on n'a pas pu mettre en evidence l'utilité de l association radioprotecteurs + agents alkylants.

SUMMARY

A quantitative investigation was carried out to establish the usefulness of radioprotector drugs in combination with alkylating agents for the chemotherapy of Ehrlich carcinoma and Sarcoma 180 *in mice. Tumoural growth, spleen weight and number of blood white cells were measured.*

The results show that a protection against the damage induced by alkylating agents on spleen and white cells was always achieved at doses of radioprotectors able to prevent also the antitumoural activity of the same alkylating agent.

Despite the large range of doses and schedule of treatment tried it was impossible to produce evidence that the combination radioprotectors + alkylating agents was useful.

ZUSAMMENFASSUNG

Eine quantitative Untersuchung ist durchgeführt worden, um die Nützlichkeit der Verbindung Strahlenschutzstoffe mit alkylisierenden Mitteln für die Chemotherapie des Ehrlich-Carcinomas und des "Mäuse-Sarcomas 180" *zu bestimmen.*

Der Tumor-Wachstumsrate, das Gewicht der Milz und die Zahl der weissen Blutzellen wurden gemessen.

Die Ergebnisse zeigen, dass der Schutz gegen die Schädigung, -welche durch die alkylisierenden Mitteln die Milz und die weissen Blutzellen betrifft- erst mit solchen Dosen der Strahlenschutzstoffe erreicht worden ist, die gleichzeitig die tumorhemmende Wirkung derselben Mittel aufheben.

Trotz der grossen Anzahl der verwendeten verschiedenen Dosierungsformeln und Behandlungsarten war es unmöglich die Nützlichkeit einer gleichzeitigen Verwendung alkylisierenden Mittel mit Strahlenschutzstoffen zu ermitteln.

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Aspects de l'Hématopoïèse en Culture du Foie de Souris

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LE MAINTIEN *in vitro* d'une intégrité dans le tissu hématopoïétique a suscité l'intérêt des hématologistes dès les premiers temps qui ont suivi l'introduction des méthodes de culture des tissus, et très nombreux furent en conséquence les travaux qui ont été consacrés à ces questions.

Les tentatives faites pour propager de la moelle osseuse de poulet ou de mammifères [1–7] n'ont pas été couronnées de succès, et la durée de survie s'est avérée limitée à quelques jours, même dans les meilleures conditions. Les techniques qui ont été utilisées, lames creuses, chambres à lamelles, suspensions, cultures sur agar, ont toujours abouti à de telles anomalies cytologiques dans le tissu que l'individualité et la différenciation cellulaires se trouvaient perdues après culture.

MacCulloch et Parker [8] ont propagé en série de la moelle osseuse humaine dans des tubes roulants, mais leur souche devint rapidement indistinguable caryologiquement et antigéniquement de cellules L (fibroblastes de Souris) [9].

Osgood a développé un système de gradient [10] grâce auquel des types variés de cellules, soit normales, soit malignes, disposées sur une lame, sont sélectivement maintenus grâce au micro-conditionnement dû au potentiel d'oxydo-réduction et aux conditions d'oxygénation. Sa technique, cependant, n'a pas d'application, et ces cultures finissent par un envahissement de type fibroblastique.

La littérature sur la culture de cellules hématopoïétiques murines est très restreinte. Billen et coll. ont maintenu de la moelle de Souris pendant des jours [11]. En dépit du fait que leur aspect typique ait été perdu, certaines cellules étaient capables de repeupler des hôtes irradiés à dose létale, mais leur lignée devint brusquement hypotétraploïde [12], et l'origine hybride possible de la souche ne peut être exclue étant donné le comportement des cellules réticulo-endothéliales de la rate [13].

Il est vrai que de telles difficultés purent être surmontées en partie par le maintien *in vivo* des cellules hématopoïétiques [14]. Le seul rapport satisfaisant concernant une hématopoïèse prolongée et active est le travail de Benevolenskaja [15] en culture de foie foetal humain. Dans un travail axé sur les macrophages et les cellules hépatiques, sur le même matériel humain, Hillis et Bang [16] mentionnent une survie de plus de deux semaines des cellules hématopoïétiques, mais ne donnent aucun détail sur le comportement de ces cellules. Les expériences que nous décrivons ici concernent la propagation du tissu hématopoïétique du foie de Souris qui utilise un système nourricier dans une méthode de culture en tubes [17–19].

MATERIEL ET METHODES

Animaux — Des foetus de 18 jours et des nouveaux-nés provenant de croisements (CBA × DBA/2) et (CBA × C57Br) ont été utilisés. Les animaux sont fournis par Gif.

Explantation — On excise les lobes hépatiques avec des ciseaux pointus et on les transfère dans une solution chaude (BSS) [20] pour éliminer le sang, puis on les coupe en cubes d'environ 0,3 mm^3 avec des ciseaux à iridectomie et des pinces. L'épaisseur de l'explant constitue un facteur de succès important, et les fragments aplatis donnent un étalement cellulaire meil-

leur et plus uniforme que ceux qui sont épais, particulièrement en leur centre. On sélectionne les meilleurs explants et on les transfère dans une boîte de Pétri remplie avec le milieu qui doit être utilisé.

Deux ou trois fragments sont alors placés dans chaque tube de culture avec une pipette Pasteur. On place ces tubes dans une étuve pendant 20 minutes pour permettre l'adhésion. On a préparé en tout cent quatre-vingts cultures. On observe souvent que les fragments placés au fond des tubes s'attachent mal, ils donnent cependant fréquemment des colonies parfaitement viables.

On retire alors l'excès de liquide que l'on remplace par le milieu (1,2 ml par tube) puis on place les tubes dans des portoirs fixes (Arthur H. Thomas Cie) à 5° d'inclinaison sur l'horizontale.

La température d'incubation est de 37°C± 0,2, et on évite de refroidir les cultures pendant leur examen au microscope.

Milieux liquides — De nombreuses combinaisons ont été essayées. La composition d'un certain nombre d'entre elles est indiquée maintenant:

A. BSS, 70%, sérum, 20%, extrait embryonnaire isogénique standardisé et conservé à 4°C, 10%;

B. Similaire au milieu A, contenant 0,007 mM de cyanocobalamine;

C. Similaire à A avec 1:700 d'un extrait médullaire [21];

D. Le milieu de base de Eagle pour cellules L (Difco) supplémenté avec 20% de sérum;

E. 70% BSS [22] contenant 0,5% d'hydrolysat de lactalbumine, 10% d'extrait embryonnaire et 20% de sérum.

On a testé l'effet de deux sérums: sérum de cheval et d'embryon de veau. Chacun de ces sérums provenait d'un seul animal et avait été prétesté, ceci pour éviter l'introduction d'un élément toxique.

Les milieux ci-dessus contiennent de la pénicilline et de la streptomycine, 80 U/ml et 0,08 mg/ml respectivement.

On a eu soin d'éviter les baisses de pH. La phase liquide était changée en totalité tous les 2 ou 3 jours. Cette phase liquide réprésente le 1/25 du volume des récipients dans ce système.

Les fluides surnageants des cultures faites parallèlement ont été souvent collectés et centrifugés à 800 tours. Le culot est ensuite étalé en frottis colorés au May-Grundwald-Giemsa.

Cytologie des cultures — La vitesse de croissance cellulaire s'apprécie facilement en utilisant un microscope ordinaire sous faible grossissement. La viabilité cellulaire est appréciée quantitativement par l'apparence transparente des cellules. Elle est également testée par l'incorporation de rouge neutre ajouté au milieu.

Pour les études cytochimiques, les cultures sont fixées directement dans le tube par le Méthanol (pureté analytique) et colorées comme les frottis en suivant le procédé de Jacobson et Webb [22] puis détachées avec soin et montées. Les spécimens bien fixés et intacts montrent des variations, suivant les types cellulaires, de l'intensité de basophilie du cytoplasme, si bien que la distinction fine entre cellules peut être facile. Cette étape est essentielle en vue de la différenciation des cellules selon leur origine. La présence de fer dans les cellules et entre les cellules est démontrée par le test de Quincke décrit par Pearse[24] après fixation à l'éthanol absolu. La présence d'acide chondroïtine-sulfurique est démontrée qualitativement selon Lison décrit par Pearse [24] sur cellules fixées au Boin. On a employé le bleu Alcian de Gurr.

Inoculation de virus — Certaines cultures ont été infectées par des sérums d'animaux porteurs de la leucémie de Charlotte Friend. On inocule les cultures avec 0,2 ml de sérum qui reste en contact avec le tissu pendant deux ou trois jours. On répète cette opération après le premier renouvellement du milieu, en diminuant d'autant la quantité de milieu frais. Le contact avec le virus dure donc 4 ou 5 jours. Des tubes témoins traités parallèlement reçoivent la même quantité de sérum isogénique normal.

RESULTATS

Après un jour de culture, on voit émerger passivement des explants provenant de matériel nouveau-né, de nombreux éléments arrondis puis une membrane épithéliale constitue la majeure partie de l'étalement. Les éléments arrondis représentent surtout des cellules de Küppfer qui migrent librement sur l'épithélium et au-delà. Au niveau des sinus sectionnés, on trouve aussi de petits lymphocytes souvent groupés, ceux-ci, cependant, disparaissent rapidement ensuite. Les cellules de Küppfer se colorent fortement au rouge neutre. Ces macrophages, pourvus d'un noyau rond, changent de forme et se distinguent bien des leucocytes et histiocytes, ces derniers possédant un noyau annulaire chez la Souris. D'autres cellules, mésenchymateuses, sont parfaitement évidentes par endroits et tendent à s'orienter de

façon radiaire (Fig. 2). Groupées en amas, des cellules possédant une coloration rouge, caractéristique de l'hémoglobine, accompagnent la membrane migratrice, particulièrement dans les spécimens de 18 jours.

A la fin de la première semaine de culture, l'examen du matériel coloré vitalement indique une extension très limitée d'un épithélium clair et de cellules plus granuleuses dont les plus périphériques accumulent souvent des inclusions réfringentes.

Les cellules épithéliales denses s'étalent en membranes et en cordons multicellulaires en épaisseur surtout vers l'intérieur lorsqu'ils prolongent les plaques hépatiques. La multiplication de ces cellules est démontrée par une activité mitotique importante, et une estimation grossière du nombre des chromosomes indique un niveau tétraploïde pour certaines de ces cellules. Des cellules multinucléées ont été observées dans certains tubes, chacune d'entre elles, contenant jusqu'à 12 noyaux, semble tirer son origine d'un type bien déterminé, soit parenchymateux, soit endothélial ou Küppfer (Fig. 1). L'étude des conditions du phénomène a été esquissée par Lambert [13]. De nombreuses cellules binucléées sont évidentes également dans les cultures de matériel foetal.

A la fin de la deuxième semaine de culture, le tableau après fixation et coloration est le suivant (du centre à la périphérie): à l'intérieur de l'explant, de l'épithélium qui n'a pas migré, des îlots hématopoïétiques et des cellules du système réticuloendothélial. L'identification des types cellulaires est rendue difficile dans les colonies épaisses. Les précurseurs hématopoïétiques des leucocytes différenciés et des cellules de la série rouge sont étroitement liés à la membrane épithéliale ou disposés entre les doigts formés par ce tissu suivant le milieu utilisé. Des précurseurs hématopoïétiques ont été conservés jusqu'à 25 jours en utilisant certains milieux (voir ci-dessous).

La lignée lymphocytaire a complètement disparu à ce moment, excepté dans les sinus intacts du centre, ce qui est dû évidemment à la perte de ces cellules dans les renouvellements de milieu comme on peut le démontrer sur frottis de ceux-ci. De plus, à ce stade, on trouve assez peu de lymphocytes dans le foie. Des histiocytes différenciés, des leucocytes et des érythroblastes accompagnent l'extension épithéliale et réticulaire, les érythroblastes cependant paraissent avoir une vie très courte; la révélation histochimique du fer montre des dépôts abondants prédominant au centre des colonies et dans la partie la plus externe, la partie moyenne étant moins marquée. Dans la zone extérieure, on différencie toujours les fibroblastes des macrophages par les dimensions et la morphologie cellulaires, et les propriétés cytochimiques, en particulier la production de chondroïtine-sulfate par les fibroblastes.

Les "band cells" sont nombreuses à la périphérie parmi les éléments réticulaires et elles survivent au moins 15 jours dans notre milieu A. On les voit alors perdre leur cytoplasme et libérer de la chromatine dans le milieu. Certaines ont été maintenues pendant des temps plus longs, en particulier celles qui se groupent. Des préparations de cellules colorées *in situ* au rouge neutre indiquent que certaines, dans le milieu C, divisent partiellement leur noyau donnant l'impression d'une amitose incomplète. Corrélativement, les cellules épithéliales montrent alors une accumulation de granules réfringeants, ce qui rejoint les observations de Barta [25]. Après deux semaines de culture, l'arrangement topographique décrit reste à peu près inchangé jusqu'à la fin des expériences: l'architecture et la distribution dans l'espace à l'intérieur de ces zones hématopoïétiques sont complexes. Les différents types cellulaires blastiques, quels que soient leur origine ou leur degré de maturation, sont éparpillés auhasard sur la couche nourricière (Fig. 3). Les érythroblastes, avec leur taille relativement importante et leur noyau particulier, très granuleux, ont peu de cytoplasme et conservent leur forme arrondie s'opposant ainsi aux promonoblastes de taille plus importante dont le noyau en forme de haricot contient trois corps basophiles importants. Les érythroblastes et leurs précurseurs sont libérés dans la phase liquide, d'autant plus que les cultures sont plus âgées, et cela peut rendre compte de leur rareté dans les cultures de plus de deux semaines. Là, une classification de 200 cellules hématopoïétiques suivant leur type, indique que les érythroblastes représentent jusqu'à 20% de la population, sans compter les macrophages. La fréquence décroissante des érythroblastes, du centre à la périphérie, rend ces estimations plutôt fragiles puisque les cellules de la série rouge sont absentes de la zone la plus externe de l'espace de migration et restent confinées au niveau de la couche nourricière.

Nous ne savons pas quel est le rôle joué par l'expansion des cellules nourricières dans la dispersion des cellules hématopoïétiques et sanguines, ni quel est le rôle joué par le comportement migratoire de ces cellules, dû à leur faible adhésion aux autres types. Nous ne savons pas non plus si les érythrocytes que l'on

trouve en suspension préexistaient à l'explantation ou s'ils se sont formés pendant la culture. L'échec observé d'une maturation des réticulocytes en érythrocytes dans les cultures de 17 jours semble indiquer que les conditions n'étaient pas réunies pour une érythropoïèse complète. Apparemment les types monoblastiques montrent une capacité d'adhésion plus importante. Ceux qui migraient trop loin, cependant, ne pouvaient survivre, et leur disparition s'annonçait par la forme bizarre du cytoplasme, le noyau s'échappant parfois de la cellule.

Aucune méthode efficace n'a pu être mise au point pour empêcher la perte des cellules hematopoïétiques dans le surnageant. Pour des raisons obscures cependant, dans plusieurs colonies, certaines restent nombreuses, même après trois semaines.

Les tentatives de classer les cellules hématopoïétiques et sanguines sur la couche nourricière et en suspension dans les mêmes cultures indiquent que les cellules les plus évoluées ont une forte tendance à passer en suspension; ceci contraste avec leurs précurseurs qui restent adhérents à l'épithélium et, pour les métamyélocytes, aux cellules réticulaires (Figs 5–8).

Des types plus mûrs migrent librement sans dommage apparent, des leucocytes surtout et de nombreux macrophages qui accumulent du fer [26] comme le révèlent les inclusions noires dans leur cytoplasme. Une maturation de métamyélocytes en polynucleaires s'observe après trois semaines, au niveau des cellules réticulaires. Ces leucocytes cependant survivent mal sur couche nourricière. Les cellules réticulaires restent dispersées au-delà de l'épithélium, et on n'a jamais observé de maturation vers la série plasmocytaire; ceci semble indiquer clairement qu'une fois qu'un stade véritable de différenciation est atteint, il est irréversible dans des conditions physiologiques.

Effets des conditions de milieu

Les différents sérums ont été incorporés et comparés dans les milieux A et E. Avec le sérum embryonnaire de veau, on obtient, semble-t-il, de moins bons résultats qu'avec le sérum adulte de cheval. Les cellules hématopoïétiques sont moins nombreuses avec le premier sérum.

L'enrichissement du milieu A avec la vitamine B12 ne semble pas avoir d'effet sur une survie prolongée des cellules hématopoïétiques et sanguines.

Le milieu C, contenant 1:700 d'extrait médullaire, est efficace pour maintenir, à la périphérie des cultures, une maturation des leucocytes et une multiplication des cellules-souche; il semble bien également que l'épithélium soit stimulé significativement. Etudiant l'effet d'un tel extrait sur des tissus embryonnaires de poulet, Astaldi et coll. ont trouvé un effet protecteur, promoteur d'une différenciation [27], fait capital si l'on admet qu'un état dynamique des constituants multiples du tissu hématopoïétique est nécessaire pour la démonstration d'un effet transformant d'un virus [28].

Le mélange synthétique de Eagle enrichi en sérum (milieu D) s'est montré incapable d'opérer une sélection de n'importe quel type cellulaire, et les images sont obscurcies par un envahissement de type fibroblastique. Toutefois, une concentration finale de 0,4 d'hydrolysat de lactalbumine (comme dans le milieu E) donne lieu à une large extension de cellules nourricières, ce qui constitue surement une condition préliminaire pour la survie des cellules hématopoïétiques. Totter a montré en effet que les cellules hématopoïétiques sont sous la dépendance du foie pour leur nutrition [29].

Les propriétés promotrices des différents milieux pour une hématopoïèse active sont résumées au Tableau 1.

Tableau 1. *Survie et multiplication en jours des cellules hématopoïétiques dans les cultures nouveau-né selon le milieu. Survie basée sur la présence de blastes sur couche nourricière et de cellules sanguines en suspension*

Milieu (avec sérum de cheval sélectionné)	Erythropoïèse	Leucopoïèse	Nombre de cultures
A	10	15	10
B	10	15	6
C	10	15	30
D	8	8	10
E	17 (zone centrale)	21 (jusqu'à 25)	50

Afin d'obtenir une estimation grossière de la cinétique de production des cellules sanguines par leurs précurseurs, c'est-à-dire pour suivre les changements quant à la maturation finale des cellules pendant 20 jours, les comptages différentiels d'éléments sur frottis étaient d'un intérêt particulier. Dans deux expériences parallèles, les résultats sont concordants et montrent que les cellules les plus mûres prédominent en suspension. Les résultats d'un comptage sur un total de 823 cellules sont résumés dans le Tableau 2. Les cellules sont classées selon Bessis [30]. Les éléments blancs éosinophiles

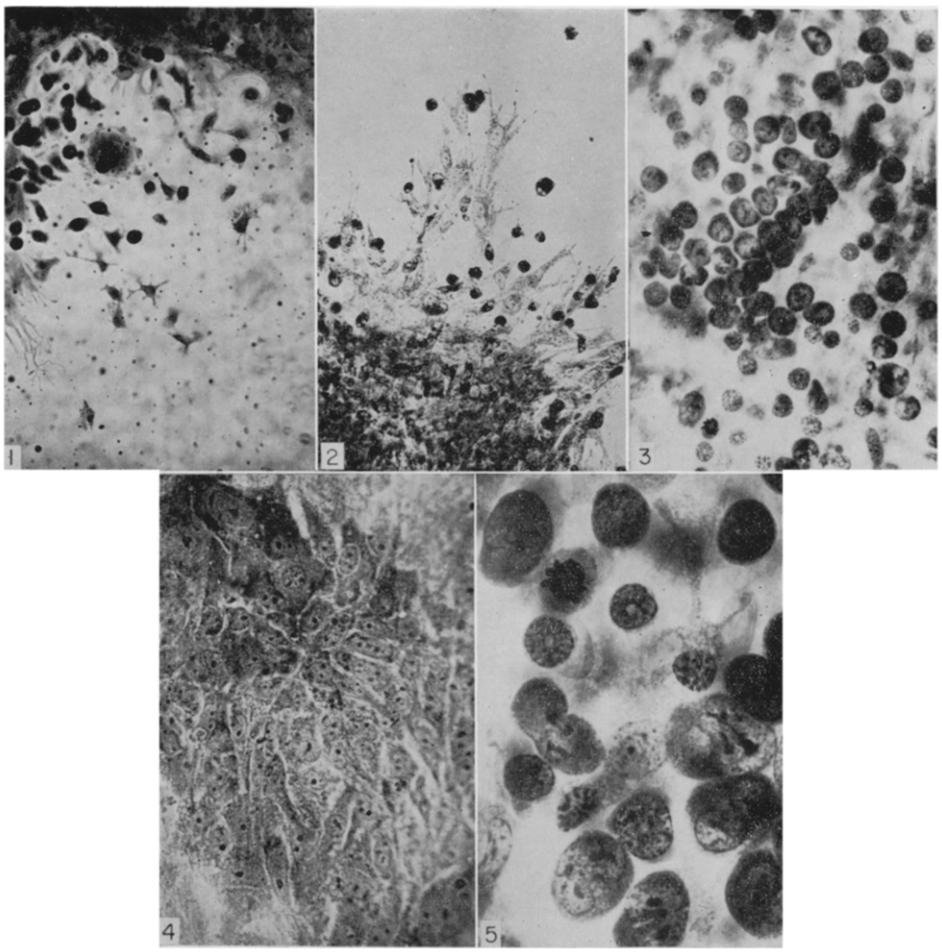


Fig. 1. *Emigration de macrophages d'un explant. Milieu A. Jour* 4 *de culture. Noter la formation d'une cellule géante.*

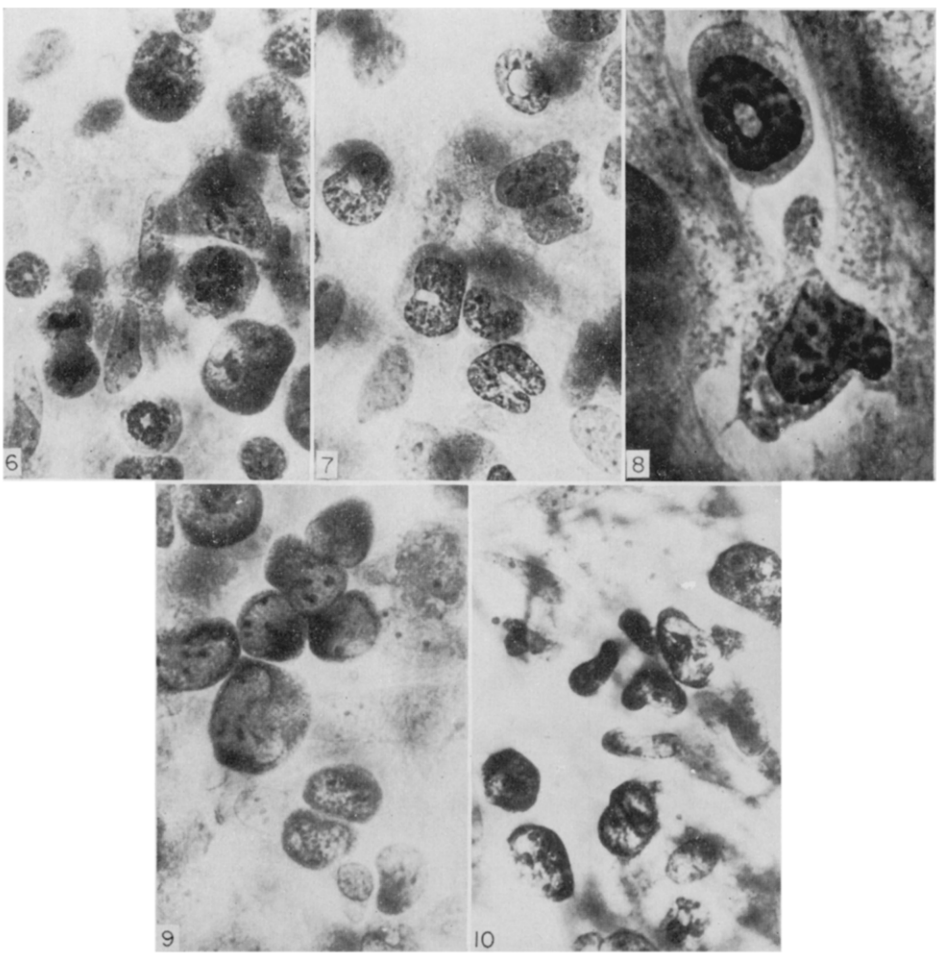
Fig. 2. *Fibroblastes et macrophages à la périphérie d'une culture de* 6 *jours. Coloration vitale avec* 1 :10 000 *de rouge neutre pendant une heure.*

Fig. 3. *Eléments blastiques variés sur couche nourricière.* 14 *e jour de culture. Colonie poussant dans un tube à deux centimètres au-dessus de la surface du milieu.*

Fig. 4. *Membrane épithéliale pure montrant l'aspect des cellules nourricières.* 14 *e jour de culture en milieu E. May-Grundwald-Giemsa.*

Figs. 5 *et* 6. *Divers blastes au* 14 *e jour de culture. Noter les mitoses.*

(to face p. 248)



Figs. 7 *et* 8. *Eléments myéloïdes migrant parmi des cellules réticulaires.* 20 *e jour de culture.*

Fig. 9. *Monoblastes et érythroblastes au 12 e jour.*

Fig. 10. *Aspect de cellules 12 jours après le début de l'infection avec le virus de Charlotte Friend. Tissu de nouveau né* (*CBA* × *DBA*2) *F*1. *Comparer avec la Fig.* 9.

Tableau 2. Répartition des types cellulaires trouvés entre 10 et 15 jours dans le surnageant des cultures en milieu E

	%
Leucocytes éosinophiles	36
Leucocytes neutrophiles	17
Leucocytes basophiles	4
Myélocytes et métamyélocytes	4,4
Monoblastes clairs	4,5
Erythroblastes jeunes	1
Erythroblastes acidophiles	10
Hématies	13
Autres blastes non classés	9,2

prédominent à 15 jours les neutrophiles à 10 jours. Les cellules dégénérées sont exclues de la statistique.

Susceptibilité virale des cultures

L'objectif essentiel de cette étude était l'essai de propagation du virus de Charlotte Friend [31] sur matériel réceptif. L'invasion rapide et extensive de la leucémie de Charlotte Friend fait de ce virus un agent idéal pour juger de l'effet de son introduction dans les cultures puisque la cellule-cible y paraît présente. Il y a là un système clos approprié avec un contrôle rigide possible des conditions expérimentales. Les premiers résultats sont positifs et les cultures paraissent souvent transformées. Douze jours après le début de l'introduction de l'agent, on observe une prédominance de cellules atypiques. Ces cellules ont un volume d'environ un quart ou un sixième de celui des grands monoblastes. Elles ont souvent une taille un peu inférieure à celle des plus petits monoblastes rencontrés dans les témoins. Le noyau est très nucléolé, et le cytoplasme très hyperbasophile, plus hyperbasophile que celui des plus grands monoblastes des témoins (Figs 9, 10). Ces cellules prédominent sur couche nourricière en tant que cellules de type hématopoïétique. Parmi ces cellules, on trouve des leucocytes et leurs précurseurs; par contre, les éléments de la lignée rouge sont absents, mais de nombreux noyaux nus à divers stades de désintégration suggèrent un effet cytopathogène sur cette lignée dans des cultures de 16 jours.

DISCUSSION

De façon classique, l'hématopoïèse est le résultat de fonctions complexes gouvernées par des interactions inductives tissulaires et humorales mal connues chez l'embryon et le nouveau-né. *In vivo*, l'hématopoïèse procède par bonds; les fonctions chez l'adulte étant relativement ségrégées dans différents sites comme la moelle osseuse ou la rate après diversification ontogénique. Cette étude a été centrée sur l'hématopoïèse essentiellement chez le nouveau-né en vue de rechercher dans quelle mesure les différentes lignées hématopoïétiques peuvent garder leur intégrité dans des conditions altérées. Il faut remarquer que la dynamique décrite diffère quelque peu de ce qui existe *in vivo* où l'hématopoïèse dans le foie cesse rapidement après la naissance. Cette situation d'équilibre entre composants multiples rend la méthode de culture très souhaitable par définition puisque multiplication et maturation s'observent corrélativement dans le tissu hématopoïétique. Nos résultats sur la durée de vie de ce tissu *in vitro* sont comparables à ce qui est connu pour l'Homme [15, 16], et la méthode permet une observation relativement facile de ces cellules. Jamais des cultures hématopoïétiques murines n'ont pu êtremaintenues pendant des temps aussi longs *in vitro*. Quand on rencontre des fibroblastes, ceux-ci représentent davantage un type cellulaire différencié présent au moment de l'explantation que l'équivalent modulé d'un blaste hématopoïétique. Nous ne pouvons pas conclure que les mitoses trouvées au niveau de la couche nourricière représentent des précurseurs hématopoïétiques qui se divisent, mais leur distribution parmi les autres types sanguines donne à croire qu'ils sont en fait des blastes en voie de différenciation. Nous n'affirmons pas non plus que l'hématopoïèse se fasse à un rythme optimum. Des études quantitatives visantà mesurer au cours du temps l'incorporation de diverses molécules marquées devraient être facilement possibles dans de telles cultures.

On a caractérisé les cellules par quelques techniques cytologiques, et le fait que le cytoplasme des cellules hématopoïétique montre une basophilie qui décroît tandis que ces cellules se différencient davantage fait penser que les antigènes présents n'ont pas joué de rôle dans la production d'anticorps. En effet, à ces stades, les animaux sont tolérants immunologiquement, et une transformation de cellules compétentes en éléments hyperbasophiles semble devoir être exclue.

Les divers types cellulaires ont très exactement la morphologie de leur prototype *in vivo*.

Enfin, l'inoculation des cultures par un virus leucémigène produisant une conversion morphologique tend à faire croire que nos cultures sont vraiment hématopoïétiques et dynamiques. Des études génétiques s'avèrent donc possibles.

Remerciements—Ce travail fait partie d'une thèse de doctorat et a été mené dans l'attente d'une subvention du National Cancer Institute (Bethesda). Je remercie les Professeurs Mathé et Amiel pour leur intérêt et le Docteur Cl. Jasmin pour son intérêt personnel constant. Mesdames M. C. Théron et E. Brugerie ont fourni une aide appréciable.

RÉSUMÉ

On a suivi l'hématopoïèse pendant des périodes allant jusqu'à 25 jours dans des cultures hépatiques de souris. On a décrit l'organisation spatiale et le comportement des cellules hématopoïétiques et on a donné les détails sur les méthodes de culture. On a pu suivre la disparition des différentes lignées cellulaires. Enfin, l'inoculation avec le virus de Friend a produit une conversion morphologique dans quelques cas.

SUMMARY

Haemopoiesis in mouse liver explants has been followed for periods up to 25 days. The spatial organization and behaviour of haemopoietic cells have been described and the details on the methods of cultivation have been given. The pattern of disappearance of the various cell lineages is outlined. The inoculation of some cultures with Friend's leukaemia virus has been shown to produce morphologic conversion in some instances.

ZUSAMMENFASSUNG

Während Zeiträume, die bis auf 25 Tage dauern, wurde die Hämatopoese in Mäusenleberkulturen Schritt für Schritt beobachtet. Die räumliche Anordnung und das Verhalten der hämatopoetischen Zellen wurden beschrieben und Näheres über die Kulturenverfahren wurde mitgeteilt. Das Verschwinden der verschiedenen Zellennachkommenschaften wurde Schritt für Schritt beobachtet. In einigen Fällen hat die Einimpfung des Friends Virus eine Wirkung ausgeübt.

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Effect of Reserpine on Immune Reactions and Tumour Growth

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IMMUNE responses may be suppressed by several types of chemical agent [1, 2]. The specific inhibition of autoimmune disease and of human homograft reactivity by immunosuppressive drugs is a major goal of experimental immunology. The clinical use of immunodepressants such as alkylating agents, antimetabolites, or tumour-inhibiting antibiotics is greatly restricted by their general toxicity, although their mode and site of inhibition within the host cell may be highly selective. Drugs such as corticoids, displaying mainly anti-inflammatory properties, suppress only some components of antibody mediated tissue injury, but fail to influence the specific immune mechanism itself [1].

Thus, any reports on immunosuppressive chemicals belonging to a new class of compounds are bound to arouse considerable interest. Recently, Draškoci and Janković and their collaborators observed a marked inhibition of several immune reactions in laboratory animals following treatment with reserpine [3, 4]. The authors suggested that this drug might interfere with "the central or peripheral pharmacological control of immune response," or that the involution of lymphoid tissues, produced regularly by sustained therapy with this compound, might provide a cellular basis for the impaired immunological reactivity. In fact, reserpine has been reported to inhibit the development of murine lymphatic leukaemias [5, 6]. As reserpine is known to exhibit strong sedative effects [7, 8] and as a consequence does affect food intake [8a, b], it seemed necessary to re-evaluate its immunosuppressive and tumour-inhibiting properties.

The effect of this drug and of some of its relatively less hypnotic analogues on body and organ weights and on the histology of the lymphoid tissues of rats and mice was examined. In addition, investigations were conducted on the influence of reserpine on haemagglutinin production to sheep red cells in mice, on delayed hypersensitivity to purified proteins and on autoimmune thyroiditis in guinea-pigs, as well as on the growth of a spontaneous mammary carcinoma in C3H mice.

The data presented in this paper indicate that these actions of reserpine are probably an indirect result of the sedative effects of the drug and a more direct result of the subsequent starvation.

MATERIALS AND METHODS

Animals — (a) Colony-bred male Swiss mice weighing 22–24 g and (b) inbred male and female C3H and C3HO mice [9] (obtained from the International Centre for the Provision and Study of Tumour-Bearing Animals, Netherlands) were maintained on mouse pellets (Nafag A.G., Switzerland). Colony-bred albino rats weighing 180–240 g were given Nafag rat cubes. Guinea-pigs of the Hartley strain, weighing 300–400 g, were maintained on Purina guinea-pig chow.

Drugs — The following compounds (CIBA) were used:

Ba-13,020	Reserpine [Serpasil®]
Su-3118	Syrosingopine [Singoserp®] phosphate
Su-7192	Propyl 18-0- (2-tetrahydropyranoyl) reserpate
Ba-4311	Methylphenidate [Ritalin®] hydrochloride

Reserpine and its analogues were dissolved in 0·65% ascorbic acid. Fresh solutions were made up daily. Methylphenidate was dissolved in water.

Treatment schedules — In experiments in which haemagglutination titres were determined, mice received subcutaneous injections of the drug on 5 consecutive days starting on the day of antigen administration (day 1).

In order to study suppression of delayed hypersensitivity to purified protein and of allergic thyroiditis, the drug was injected intraperitoneally into guinea-pigs daily for 7 days, starting on the day preceding antigen administration. Rats used for histology were injected intraperitoneally with the drug on 14 consecutive days. Mice bearing spontaneous mammary tumours were injected with the drug intraperitoneally daily during the first week and every other day during the second and third week (except over the week-ends), eleven injections being given altogether.

Starvation — In some experiments, mice were kept individually in single cages on a wire net. Food was withdrawn as from the day of antigen administration. Water was given *ad libitum.*

Force feeding — Groups of reserpine-treated mice were fed five times daily by stomach tube a mixture of commercial baby foods (approximately 200 mg Elonac® (Guigoz SA, Switzerland), and 25 mg Humana® (Schweizerische Milchgesellschaft, Hochdorf, Switzerland) per day. This diet was supplemented by a daily intraperitoneal injection of 0·5 ml Aminosol Vitrum® (Hausmann).

Since mechanical force-feeding of the reserpine-anorectic mice was impractical over the 3-week period necessary for observation in the tumour experiments, the animals were stimulated for periods of about 30 min by daily intraperitoneal injections of 5–10 mg/kg methylphenidate (by analogy with the procedure of Kurland [10]). The latter compound by itself had no effect on tumour growth, but enabled the animals to obtain some food and water during the limited periods of alertness due to methylphenidate.

Body and organ weights — In most experiments, total body-weights were recorded daily. After the animals had been sacrificed, weights of thymus and spleen, and in some groups, of heart, kidney, liver, and testes were recorded.

Histology — Animals were killed for histological studies at various intervals after the onset of drug treatment. Tissues were fixed in Carnoy's or Bouin's fluid, cut at 5μ and stained either with haematoxylin and eosin, or with Sudan II.

Haematocrit values were determined by standard haematological procedures.

Haemagglutinin production — Haemagglutinins to sheep red blood cells (SRBC) were determined with the aid of a previously described technique [11]. Briefly, mice were immunized with a single intraperitoneal injection of SRBC and bled 4 and 9 days later by orbital puncture Sera were titrated using a Takatsy microtitre kit with titres expressed as reciprocals of the end point dilution (powers of the base 2). Controls matched for body weight and receiving appropriate injection of solvent were included in all experiments.

Delayed hypersensitivity — For the induction of delayed hypersensitivity, guinea-pigs were injected in the footpads with 5μg hen egg albumin in incomplete Freund's adjuvant. The animals were tested intracutaneously with 5μg hen egg albumin in saline containing 1% guinea-pig serum on days 6 and/or 7 to determine the extent of delayed hypersensitivity and on days 13 and 14 to measure Arthur-type hypersensitivity. Reactions were observed at 4, 18 and 24 hr [12].

Autoimmune thyroiditis — Guinea-pig thyroglobulin was prepared by the modified [13] method of Derrien *et al.* [14]. On the second day of drug administration, the guinea-pigs were injected in the footpads with 2·5 mg thyroglobulin in incomplete Freund's adjuvant. Four to six weeks later, the animals were challenged in the footpads with 100 μg guinea-pig thyroglobulin in Freund's complete adjuvant containing 2 mg tubercle bacilli. At irregular intervals thereafter, the guinea-pigs were skin-tested for delayed or Arthus-type hypersensitivity, and bled for determination of antithyroid antibody by passive cutaneous anaphylaxis [15], and thyroid glands were extirpated for histological examination.

Spontaneous mammary tumours — A modification of the test system described by Le Page *et al.* [16] was used. Briefly, groups of fifteen

animals with single primary tumours of comparable size (5–15 mm dia.) were treated and observed during a 3-week period. The average weight of the animals was recorded weekly and the growth of the tumours was measured simultaneously in two diameters by calipers. Both values were compared with those obtained in control animals injected with solvent.

Statistical analysis — Results were analysed by Student's "t"-test.

RESULTS

Body and organ weights — Prolonged administration of high doses of reserpine and some of its analogues in mice and rats produced diarrhoea and a soporous state, which was associated with a reduction in food intake, a rapid deterioration in the animal's general condition, and a dose-related increase in mortality.

Reserpine-induced reduction of body and organ weights was dose-dependent (Fig. 1). During the early days of treatment, as seen on day 4, thymus weights were more depressed than spleen weights. Later, as seen on day 9, spleen and thymus showed a comparable weight loss. Starvation, in the absence of drug administration, produced a decrease in weight of the same order of magnitude as that seen following daily administration of the highest dose (1 mg/kg per day) of reserpine tested (Fig. 1).

Histologically, typical alterations could be observed on day 4. Administration of 0·3 and 0·4 mg/kg reserpine subcutaneously per day induced marked narrowing of the thymic cortex with a partial depletion of lymphocytes which accumulated in peripheral parts of the medulla. Spleens and lymph nodes retained their normal structure.

Daily injection of 0·5 mg reserpine or more per kg subcutaneously (causing a reduction of

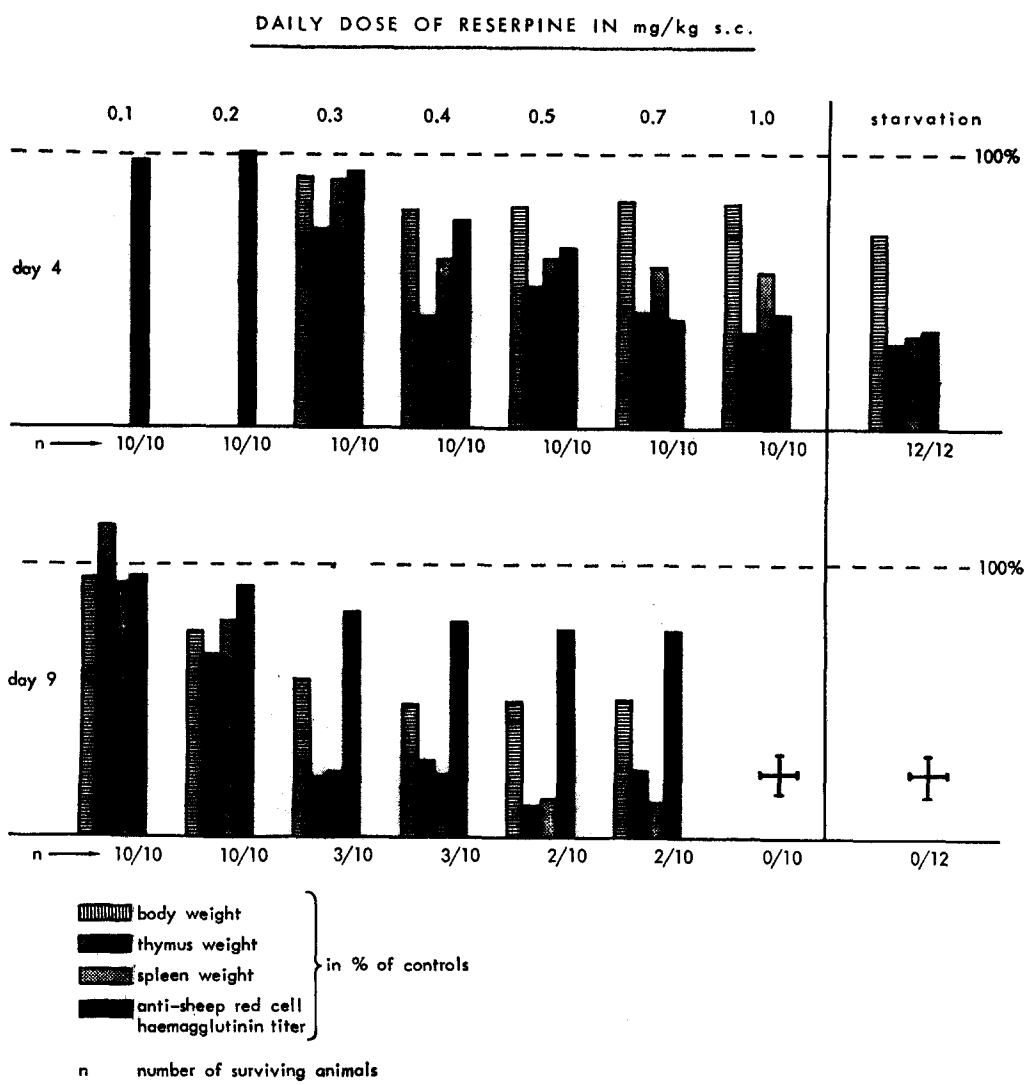

Fig. 1. *Effect of reserpine or starvation on weights and haemagglutinin formation in mice.*

3/4–4/5 in thymic weight) was associated with complete lymphoid depletion of the thymic cortex, which became wholly epithelial and contained scattered pyknotic cells, fragmented nuclei, and cellular debris. On the other hand, the medulla was packed with lymphocytes, thus displaying a reversal of the normal thymic architecture (Figs. 2A and 2B).

The spleens showed only moderate depletion of the white pulp, with dissociation of lymphoid follicles, as well as hyperplasia of red pulp and reticular elements (Figs. 3A and 3B). In the lymph nodes, a moderately decreased cellularity was apparent (Figs. 4A and 4B).

Starvation alone produced qualitatively and quantitatively the same histological changes as the highest doses of reserpine employed (Figs. 2C, 3C and 4C).

On day 9, histological alterations of the lymphoid tissues were even more pronounced. As little as $5 \times 0{\cdot}3$ or $0{\cdot}4$ mg/kg reserpine subcutaneously caused complete thymic involution, extensive reduction of the lymphoid follicles of the spleen, and marked hypoplasia of the lymph nodes (Figs. 2D, 3D and 4D).

Since the degree of weight loss seemed to be linked to the drug-induced sedation and thus to the reduction in food intake, force feeding of reserpine-treated mice was attempted. Body, thymus, and spleen weights of the force-fed groups were somewhat higher than those of animals treated with reserpine alone (Fig. 5). However, the differences were not statistically significant.

Essentially identical histological alterations and weight changes were obtained when mice were treated with sublethal doses of the reserpine analogues Su-3118 and Su-7192. Again, rats given daily intraperitoneal injections of 1 mg/kg reserpine over a 2-week period showed a similar involution of their lymphoid tissues, as well as of heart, liver and kidneys.

Haemagglutinin production — The effect of various dosages of reserpine on haemagglutinin production in mice is shown in Fig. 1. In view of the limitations of the method, only a reduction in titre of more than 25% was considered to be significant [11]. Thus, reserpine doses of $0{\cdot}5$ mg/kg per day or more were necessary to suppress antibody formation on day 4 (titre $2{\cdot}1 \pm 1{\cdot}3$ vs. $5{\cdot}0 \pm 1{\cdot}0$ in control mice). However, starvation alone (Fig. 1) produced a similar reduction (titre $1{\cdot}8 \pm 1{\cdot}6$). On day 9, the titres of all surviving mice were again within the range of that of the controls ($7{\cdot}2 \pm 0{\cdot}7$).

A similar pattern was observed with the reserpine analogues Su-3118 and Su-7192 which are both substantially *less* soporific than reserpine itself. Only near-lethal doses, causing marked sedation and diarrhoea, produced a temporary reduction in haemagglutinin titres on day 4. On day 9, all the survivors from the experimental groups (1/5 in the case of Su-3118 and 5/5 in that of Su-7192) showed normal antibody titres.

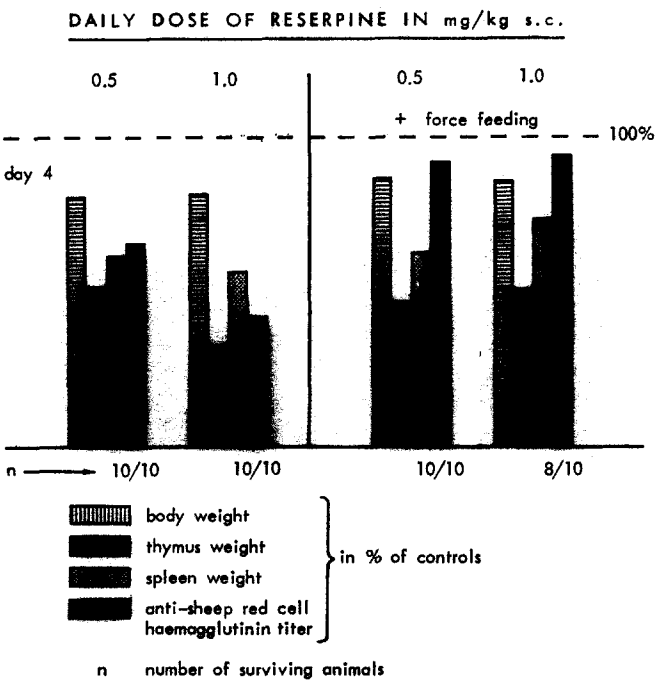

Fig. 5. Effect of reserpine on weights and haemagglutinin formation in force-fed mice.

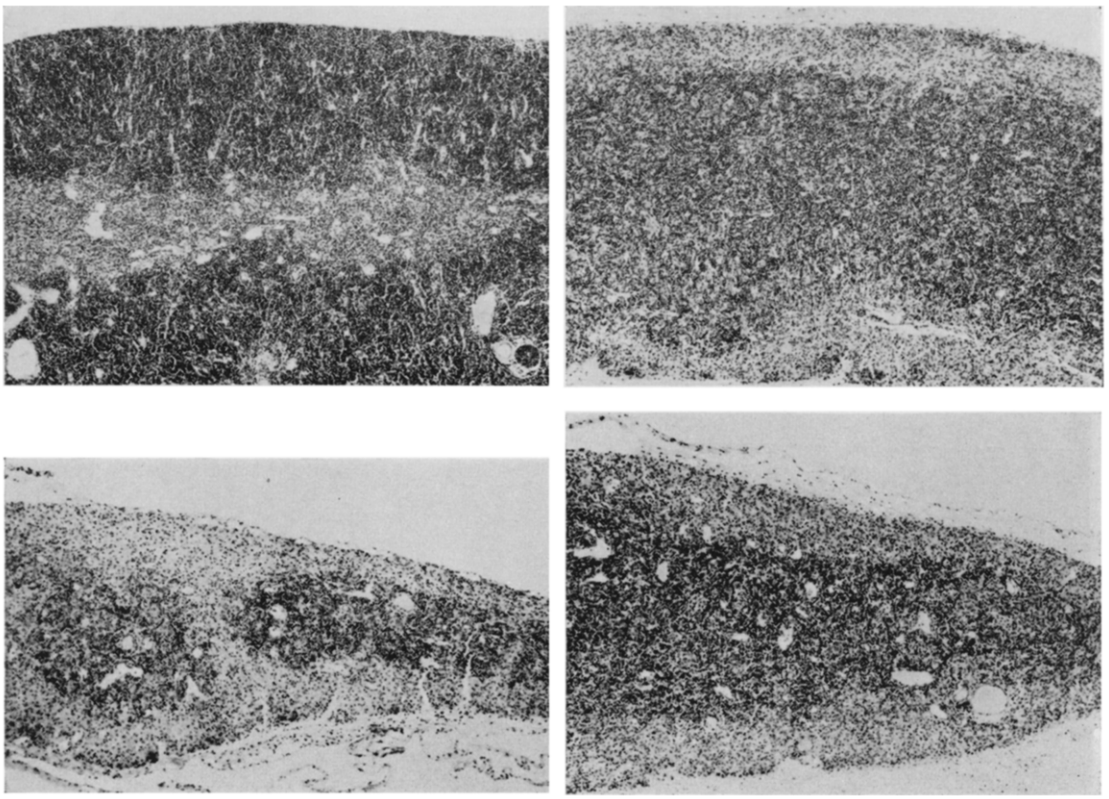

Fig. 2. *Mouse thymus* (*magnification* 26×, H.E.)

A: Control: Typical correlation between predominantly lymphoidal cortex and mainly reticulo-epithelial medulla

B: Reserpine 1 mg/kg per day (4×) s.c. *day* 4:
C: Starvation: day 4:
D: Reserpine: 0·4 mg/kg per day (5×) s.c. *day* 9:

} *Cortex depleted of lymphocytes, relative increase in lymphoid cells in medulla.*

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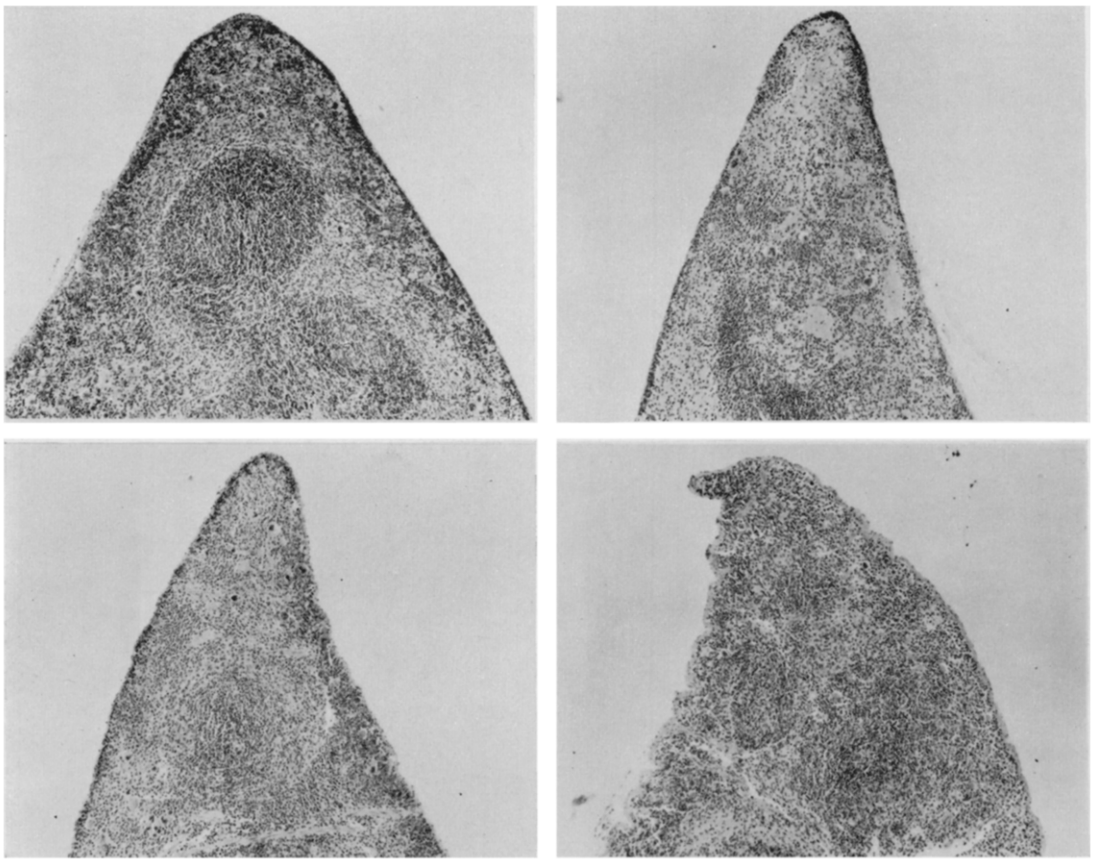

Fig. 3. *Mouse spleen* (*magnification* 26×, H.E.)

A: Control: *Red and white pulp in typical proportions*
B: Reserpine: 1 mg/kg per day (4×) s.c. *day* 4: } *Diminution of lymphoid follicles, increase in*
C: Starvation *day* 4: } *haemopoietic activity, reticular hyperplasia*
D: Reserpine 0·4 mg/kg per day (5×) s.c. *day* 9: *Complete loss of follicular structure, marked lymphoid depletion.*

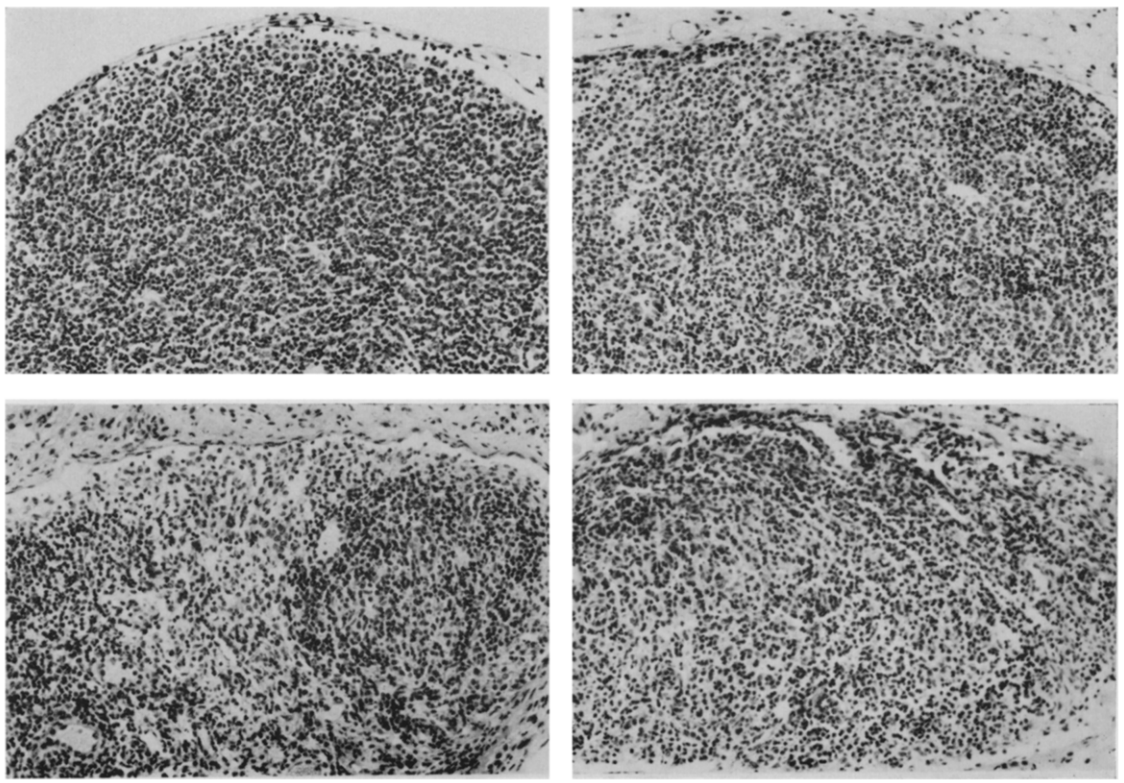

Fig. 4. *Mouse inguinal lymph-node* (*magnification* $\times 60$, H.E.)

A: Control	*Cortical area with densely packed lymphocytes*	
B: Reserpine	1 mg/kg per day ($4\times$) s.c. *day* 4:	*Cortical area showing moderate reduction in small lymphocytes*
C: Starvation	*day* 4:	
D: Reserpine	0·4 mg/kg per day ($5\times$) s.c. *day* 9:	*Substantial lymphoid depletion of whole lymph-node.*

Force feeding effectively offset the temporary immunosuppression produced by reserpine (Fig. 5). The differences in titres between reserpine-treated mice with and without force feeding were statistically significant ($p < 0{\cdot}05$ for mice given 0·5 mg/kg per day, $p < 0{\cdot}01$ for animals treated with 1·0 mg/kg per day).

All haematocrit values on day 4 of reserpine-treated, starved and force-fed animals were within the range of control animals.

Skin hypersensitivity to hen egg albumin. Autoimmune thyroiditis

The results are summarized in Table 1. Guinea-pigs treated with the maximal tolerated dose of reserpine (0·3 mg/kg per day) developed delayed and Arthus responses to hen egg albumin that were virtually identical to those in untreated controls.

Similarly, this drug had no effect on the

Table 1. *Effect of reserpine on hypersensitivity in guinea-pigs*

	Skin reactions to hen egg albumin			Autoimmune thyroiditis	
Treatment	Delayed*	Arthus	Delayed*/ Arthus	PCA	Cellular infiltration
Reserpine 0·3 mg/kg per day	8/12†	7/7	5/7	5/7	5/7
Controls	9/12	8/8	8/10	8/10	8/10

*Skin reaction, 10 mm or more in diameter.
†Numerator: number of animals with positive responses.
Denominator: number of animals tested.

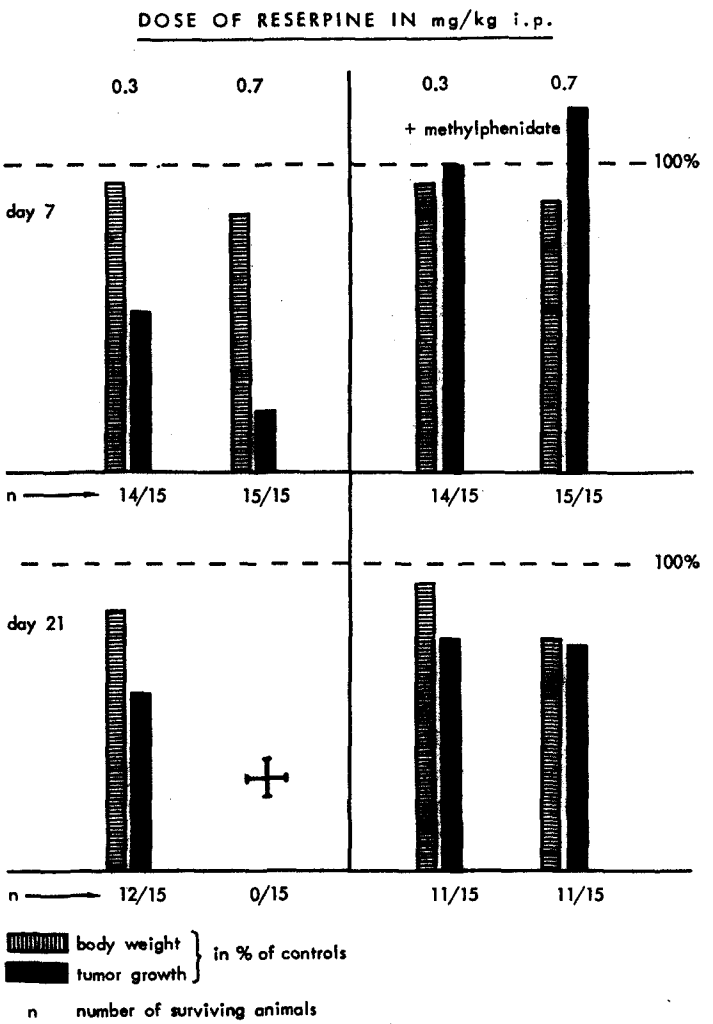

Fig. 6. *Effect of reserpine on growth of spontaneous mammary carcinomas in C3H-mice.*

development of skin hypersensitivity, circulating antibody, and cellular infiltration in autoimmune thyroiditis.

Spontaneous mammary tumours — The effect of reserpine alone and in combination with methylphenidate hydrochloride on body weight and on the growth of primary breast tumours in C3H mice is summarized in Fig. 6. Administration of 0·3 mg/kg reserpine alone (left side of Fig.6) caused almost a 50 per cent inhibition of tumour growth and 0·7 mg/kg a more than 80 per cent inhibition after 1 week of treatment.

With the lower dose, this inhibitory effect remained constant throughout the 3-week period of observation. Mice treated with 0·7 mg/kg died before the end of the second week of the experiments. At both dose levels of reserpine, the animals became increasingly more somnolent.

The tumour-inhibiting effect of reserpine was virtually eliminated in mice injected simultaneously throughout the experiment with 5–10 mg/kg per day methylphenidate hydrochloride (see right side of Fig. 6). Under these conditions, 0·3 mg/kg reserpine had no effect after 7 days of treatment and caused a tumour inhibition of less than 25% after the third week. This degree of inhibition is considered not to be significant. Mice injected with 0·7 mg/kg reserpine and stimulated simultaneously showed larger tumours than their controls after the first week. These differences, however, are within the limits of experimental variations. Moreover, such animals survived the 3 week period of observation, at the end of which time the final inhibitory effect of reserpine on tumour growth and body weight was virtually identical.

DISCUSSION

(1) Prolonged treatment of rats and mice with reserpine was associated with a dose-dependent involution of lymphoid structures. Since the lymphatic organs play a central role in immune reactions, the belief may be held that lymphoid depletion *per se* may provide a cellular basis for immunological impairment [3, 4]. However, the data presented in this paper clearly show that mice with only moderately reduced peripheral lymphatic tissue, as is found early on during a course of reserpine administration, showed a highly significant impairment of their immune response. On the other hand, at a later stage, animals presenting extreme involution of their lymphoid tissues did not show significant depression of their capacity to form antibodies.

As thymectomy in adult life is not associated with any immediate impairment of immune reactivity [17–19], mere thymic involution cannot be expected to interfere directly with an animal's immune capacity. Furthermore, it has been demonstrated that correlation is poor between the cellularity of peripheral lymphoid tissues and the immunological potential of neonatally thymectomized mice bearing thymus tissue in diffusion chambers [20]. Thus, the findings in this paper further support the concept that immunological reactivity depends rather upon the *qualitative* than the *quantitative* state of the lymphatic system [21].

(2) The immunosuppressive effect of reserpine and of the analogues studied in this report appears quite marginal. Only very high dosages within the sublethal range were shown to lower haemagglutinin production against heterologous red cells in mice. On the other hand, both skin hypersensitivity towards hen egg albumin and development of autoimmune thyroiditis in guinea-pigs, which are known to be suppressed by specific [22] and non-specific [23, 24] methods, were not influenced at all. Furthermore, the immune response was only temporarily suppressed by reserpine during the early log phase [11] of antibody formation (day 4) and did not even last until day 9, when normal antibody titres developed.

In most of the experimental groups receiving high doses of the drug, a proportion of the animals died between the 2 days of antibody determination. The question therefore arises as to whether the apparent normalization of the immune response was simply due to a selection of resistant mice. However, in experments using the reserpine analogue Su-7192, the same individual animals showed reduced antibody titres when bled on day 4, but did no longer differ from their controls on day 9. Hence, this temporary suppression of the immune response may reflect either a mere delay in, or else a rapid recovery of, antibody production.

(3) The relevance of reserpine's action on lymphoid depletion and immunosuppression becomes even more doubtful when due account is taken of the pharmacological effects [7–8b] of this group of drugs. Thus, significant impairment of the immune response was regularly associated with marked sedation and the development of diarrhoea, both of which necessarily interfered with normal food and water balance. It was therefore important to

investigate at the same time not only the effect of starvation alone, but also the countereffect of force feeding during the treatment with reserpine. Our data show that both reduction of body weight and involution of lymphoid tissue with immune suppression were produced by mere starvation of the animals. After mechanical force feeding, inhibition of antibody production by reserpine could no longer be detected.

(4) Similarly, the tumour-inhibiting effect of reserpine appears to be directly linked to its sedative action [25–28] and the resultant anorectic state, for this effect could be successfully neutralized by simultaneous administration of methylphenidate hydrochloride, which acts as a central stimulant.

RÉSUMÉ

Le mécanisme d'action de l'activité immuno-suppressive et cytostatique de la réserpine a été étudié.

Chez des rats et des souris, l'administration prolongée de très hautes doses s'est accompagnée de l'apparition d'un état stuporeux, avec chute considérable du poids corporel et du poids des organes, ainsi que de profondes modifications dans la structure des tissus lymphoïdes. Cependant, la privation de nourriture, à elle seule, a produit une involution comparable des tissus de la rate et des ganglions lymphatiques.

En dépit d'une extrême déplétion du tissu lymphoïde, les souris traitées à la réserpine ou à l'un de ses dérivés, ont finalement développé des quantités d'hémagglutinine aux hématies de mouton, proches de la normale. Au cours de la phase de production des anticorps, on a observé un effet immuno-suppressif, dépendant de la dose, de manière significative, et qui a pu être reproduit aisément par simple privation de nourriture. D'autre part, un gavage préalable a empêché l'action suppressive de la réserpine. Chez les cobayes, la réserpine n'a eu aucune influence sur le développement, soit d'une hypersensibilité cutanée à l'égard de l'albumine des poulets, soit d'une thyroïdite d'auto-immunisation.

Les doses maximales de réserpine tolérées, administrées à des souris porteuses de cancers mammaires spontanés, avaient un léger effet cytostatique qui pourrait être en relation avec l'importancee de la perte de poids et qui est contrarié efficacement par un stimulus central.

Il apparaît que les faibles propriétés immuno-suppressives et cyto-inhibititrices de la réserpine ne semblent être déclenchées que par un mécanisme indirect.

SUMMARY

The immunosuppressive and tumour-inhibiting actions of reserpine were investigated.

In rats and mice, prolonged administration of very high doses was associated with the development of a soporous state, with a considerable drop in body and organ weights, and with profound histological changes in lymphoid structures. However, starvation alone produced a comparable involution of thymus, spleen, and lymph nodes.

In spite of extreme lymphoid depletion, mice treated with reserpine or some of its derivatives eventually produced near normal amounts of haemagglutinins to sheep red cells. During the log-phase of antibody production, a significant dose-dependent immunosuppressive effect was observed, which was easily reproduced by mere starvation. Moreover, force feeding prevented the suppressive action of reserpine. In guinea-pigs, reserpine did not have any influence on the development either of skin hypersensitivity to hen egg albumin or of autoimmune thyroiditis.

Maximal tolerated doses of reserpine given to mice bearing spontaneous mammary carcinomas had a slight tumour-inhibiting effect, which could be correlated with the extent of body weight loss and antagonized effectively by a central stimulant.

Hence, the minor immunosuppressive and tumour-inhibiting properties of reserpine seem to be initiated only by an indirect mechanism.

ZUSAMMENFASSUNG

Die immunosuppressiven und tumorhemmenden Eigenschaften von Reserpin wurden auf ihren Mechanismus hin untersucht.

Bei Ratte und Maus führte längerdauernde Verabreichung hoher Dosen bald zu einem soporösen Zustand mit beträchtlichem Abfall des Körpergewichts und einer weitgehenden Involution der lymphatischen Organe. Blosses Hungern hatte ähnliche Veränderungen von Thymus, Milz und Lymphknoten zur Folge.

Trotz der hochgradigen lymphatischen Atrophie bildeten Reserpin-behandelte Mäuse schliesslich fast normale Haemagglutinintiter gegen Schaferythrocyten. Nur während der initialen log-Phase der Antikörperbildung wurde unter Reserpin vorübergehend eine signifikante dosisabhängige Titerverminderung beobachtet. Ein vergleichbarer Titerabfall fand sich freilich auch bei Hunger-Kontrollen. Ferner verhinderte gleichzeitige Zwangsfütterung die ohnehin nur bescheidene immunosuppressive Wirkung von Reserpin.

Beim Meerschweinchen vermochte Reserpin weder die allergischen Hautreaktionen gegenüber Eialbumin noch die Entwicklung einer Autoimmunthyreoiditis zu unterdrücken.

Schliesslich zeigte Reserpin einen gewissen Hemmeffekt auf das spontane Mammakarzinom der C³H-Maus, der freilich wiederum dem Körpergewichtsverlust entsprach und überdies durch Gabe eines zentralen Stimulus aufgehoben werden konnte.

Die Befunde sprechen für einen indirekten Wirkungsmechanismus des Reserpin in den untersuchten Modellen.

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A Study of Adrenal Function in Seventy-six Consecutive Cases of Bronchial Carcinoma*

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INTRODUCTION

THE occasional association of bronchial carcinoma, especially of the oat cell variety with clinical and laboratory manifestations strikingly similar to those of Cushing's disease has been reported frequently [1]. It has been attributed to the secretion by the tumour of proteins with ACTH-like activity. The argument in favour of this concept was the isolation of corticotrophin-like material from the tumour in cases displaying the signs of hypercorticism although direct secretion by the tumour and the presence of chemically pure ACTH were never demonstrated in these patients [2, 3].

Clinically, the hypercorticism associated with "non-endocrine" tumours is very pronounced with marked biochemical changes in blood and urine [4]. It appears to be quite different from the moderate hypercorticism observed commonly in acute or subacute diseases, such as could be expected in bronchial carcinoma.

The present study of adrenal function, in seventy-six consecutive patients with bronchial carcinoma, was undertaken in order: 1. to discover, in a prospective fashion, cases of severe hypercorticism in bronchial carcinoma and thus evaluate its incidence; 2. to evaluate the effect of bronchial carcinoma, considered as a stress agent, on adrenal function; 3. to discover whether there existed also cases of moderate but definite hypercorticism which could not be explained by the normal adrenal hypersecretion due to stress in addition to the rare patients described in the literature with severe and acute hypercorticism due to an ACTH-secreting bronchial carcinoma.

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METHODS

Seventy-six consecutive patients were studied on the wards of the Institut Jules Bordet between April 1963 and July 1965. The diagnosis in nine cases in which no biopsy is available was "probable bronchial carcinoma" and was based on clinical, X-ray and endoscopic evidence of a bronchial tumour. In the remaining sixty-seven patients, the diagnosis was confirmed by a positive biopsy: there were twenty-five cases of oat cell carcinoma, four anaplastic carcinomas and thirty-eight adeno- or squamous cell carcinomas.

The exploration of adrenal function was done on admission (or on successive admissions) prior to any specific chemotherapy; no medication known to alter hormonal measurements was given. Plasma 17-hydroxycorticosteroids as well as urinary 17-hydroxycorticosteroids and 17-ketosteroids were measured at least once, usually twice on consecutive days. An intravenous ACTH test was performed in thirty-two patients taken at random. In practically every patient, liver function tests, electrolytes, blood sugar, urine sugar and PBI were determined repeatedly.

Plasma corticosteroids were measured by the

method of De Moor and co-workers [5]; by this method, the normal values are 21·9γ± 4·76 per 100 ml; in our studies, values higher than the mean by more than two standard deviations, i.e. over 31·4γ per 100 ml, were considered as abnormally elevated.

Urinary corticosteroids were determined by a slight modification of the method of Porter and Silber [6] as follows: 2 ml urine + 1000 units of β glucuronidase + 2 ml acetate buffer pH 4·5 were incubated during 24 hr at 37°C; steroids were extracted with 23 ml methylene chloride and washed successively with 5 ml 0·1 N NaOH and 5 ml water a 10-ml aliquot of the washed methylene chloride was extracted with 0·5 ml of the colour reagent and another 10 ml was extracted with a blank reagent. In seventy-nine controls we found a mean excretion of 4·9 mg per day ±1·85 mg. Here again, any value higher than the mean by two standard deviations, i.e. over 8·6 mg per day was considered abnormal.

Urinary ketosteroids were measured by the method of Drekter and co-workers [7].

The intravenous ACTH test was performed as described by Christy and associates [8]. These authors measured plasma corticosteroids by the method of Porter and Silber and their limits of normal response were 35–55γ per 100 ml of plasma. In our series, any response between 55 and 60γ per 100 ml was still considered as borderline normal in order to avoid discussion about differences of methodology for measurements of plasma corticosteroids; since De Moor *et al.* demonstrated that the results by fluorometry and by the Porter and Silber reaction are very similar, our percentage of excessive responses to ACTH may thus be underevaluated.

The methopyrone tests were performed according to Liddle *et al.* [9].

The adrenals were inhibited by administering 2 mg of dexamethazone during 48 hr; urinary steroids were measured the day before and the second day of dexamethazone administration.

RESULTS

A. *Hypercorticism**

There was no clinical evidence of hypercorticism in any patient.

The most frequent observation was an abnormally high urinary excretion of 17-hydroxycorticosteroids. Such an excessive excretion was found in fifteen patients (Tables 1 and 2) but was usually moderate, ranging from 8·8 to 20·1 mg per 24 hr; the mean of these elevated values was 11·4 mg with a standard deviation of 2·8 mg. However the mean corticosteroid excretion for the entire series of lung carcinomas (124 measurements in sixty-eight patients) was 4·8 mg per day which is not different from the mean value found in controls.

High concentrations of 17-hydroxycorticosteroids in plasma were encountered less

*The term of hypercorticism is used in this paper in the widest sense and somewhat arbitrarily when, at least once, 17-hydroxycorticosteroids are increased in plasma and/or in urine; speculations concerning the possible interference of abnormal protein bindings in plasma or abnormal extra-adrenal metabolism are avoided.

Table 1. *Increased* 17*OH corticosteroid excretion or plasma concentration*

Patient	Test	Plasma 17OHCS	Urinary 17OHCS	Urinary ketosteroids	Histology	Survival time (>or<3 months)	Liver metastases
			—Increased 17OH corticosteroid excretion				
—Va..		24γ	15·6 mgr	18·5	Adeno C	<	—
—Bo..		25–21	12–9·1	4·8–3·5	Oat cell C	<	—
—Hu..			11·4	13·3	Oat cell C	<	—
—Sch..		26–28	11·4–9·5		Squamous	<	—
—De C..		24·2–12·6	10·2–6·4	17·2–6·4	Oat cell + myeloma	<	—
—Mi..		21–14·6	9·8–7·5	9·5–6·6	Squamous	<	—
	ACTH	23→72					
—Pi..		19·5–20·5	9·5–7·7	5·5–4·4	Adenocarc.	>	—
	ACTH	25→90					
—Mie..			9·3–8·5	4–4	Oat cell	<	+++
	ACTH	32→58					
—Leo..		27·8	8·1		Oat cell	<	—
—De H..		27	9	4·7	Squamous	>	—
			—Increased plasma 17OH corticoids				
—Lie..		30·2–33·7	3·1–3·2	1·6–2·1	Adeno C	>	+++
	ACTH*	47·5→>100					

*2 days before death.

Table 2. Increased plasma and urinary 17OH corticoids

Patient	Test	Plasma 17OHCS	Urinary 17OHCS	Urinary ketosteroids	Histology	Survival time (>or<3 months)	Liver metastases
—Da..*		24	13·4	10·6	Adenocarc.	7 months	—
		27·5–32·5	1·7–1·4			4 weeks	+++
—Sa..†		33·1–28·7	8·8–9·5	9·2–6·4	Squamous	6 weeks	+
	ACTH	42→91					
	ACTH	25·6→108·7					
—Ho..		43–47	15	12·1	Oat cell	8 days	—
—Tr..		34–37·5	13·2–9	6·3–11·9	Oat cell	6 weeks	+
	ACTH	35·6→76·2					
	Dexamethazone		13·2→6·4	6·3→3			
	SU 4885		9→35·4				
—Thi..‡	ACTH	47→85			Oat cell	4 months	+
			10–16·3	4·7–6·8		6 weeks	
	ACTH	49·3→145					
	Dexamethazone		12·1→7·4				
	SU 4885		11·1→28				
		40·5	20·1			1 week	

*Autopsy: left adrenal destroyed by metastases; right adrenal = 10 g preserved.

†Autopsy: each adrenal weighed 20 g — no metastases

‡1 week before death: no diurnal variation of plasma corticoids: 8 a.m. = 40·5 2 p.m. = 39·2
8 p.m. = 33·7 2 a.m. = 42·5

frequently (Table 2) and when present, were associated with increased corticosteroid excretions, except in patient Lie (Table 1). Six patients had at least once plasma corticosteroids higher than 31·4γ per 100 ml; the mean and standard deviation of these elevated values was 39·6 ± 6·3γ per 100 ml. However the mean plasma concentration found in the entire series of bronchial carcinomas (145 measurements in sixty-five patients) was 21·4γ per 100 ml and quite similar therefore to that found in controls.

The patients with high plasma and/or urinary 17-hydroxycorticosteroids maintained until their death normal electrolyte and blood sugar values. Moreover, in patient Thi (Table 2), shortly before death, blood sugar still responded normally to a test dose of insulin after 4 months of exhibiting these high plasma corticosteroid values.

Response to SU 4885 and to dexamethazone: patients Tr. and Thi. (Table 2), the two cases of oat cell carcinoma with high plasma and urinary corticosteroids and survival longer than a week, responded well to SU 4885 and to dexamethazone.

Response to ACTH: Excessive response to ACTH was found in 31 per cent of the patients tested (Tables 1, 2 and 4). One of the highest responses was observed in patient Sa. (Table 2) whose adrenals at autopsy were quite enlarged, weighed 20 g each and were not invaded by metastases. In four patients, an excessive response to ACTH was the only abnormality (Table 4). The seven patients with high urinary excretion and/or high plasma concentrations of 17-hydroxycorticosteroids and who were given a test infusion of ACTH all had an exaggerated response (Table 1 and 2) except patient Mie. (Table 1) whose response was at the upper limit of normality.

B. Hypocorticism

In only one patient, Van C. (Table 3) was there clear-cut clinical and laboratory evidence of hypocorticism. This hypocorticism developed rapidly, after a few days of hospitalization, with asthenia, orthostatic hypotension and vomiting. At this time, blood sugar was 65 mg per 100 ml, hyponatremia was marked: 118

Table 3. Low plasma corticoids

Patient	Test	Plasma 17OHCS	Urinary 17OHCS	Urinary ketosteroids	Histology	Survival time (>or<3 months)	Liver metastases
–Van C..		1–5·6	2·4–2·9	0·5–0·3	Oat cell	<3 months	+
	ACTH	5·6→28					(Found at
	SU 4885		3·7→3·6	1·2→0·9			autopsy but normal liver tests)

Table 4. Response to ACTH

Patient	Plasma 17OHCS	ACTH Test	Urinary 17OHCS	Urinary ketosteroids	Histology	Liver metastases
		Low response — (four out of thirty-two patients)				
—Wi..	17·2–18·1	11·8→30	4·5–5	4·1–2·3	Adenocarc.	—
—Mi..	15–18·7	15·8→31·6	4·8–4·4	6·7–5·4	Squamous	—
—Hi..	15·5–16·5	14·3→31·4	4·7–5·7	6·4–8·9	Oat cell	—
—Vand..		11·2→32·5	3·3	3	Squamous	—
		Excessive response — (ten out of thirty-two patients)				
—Schai..	26·8	14·2→66	6·4–5·9	6·6–1·2	Oat cell	+
—Cl..	25–20	27→66·5	3·7–3·2	7·9–5	Oat cell	—
—Ba..	26·2–28	24·2→67·4	5·7–3·9	6·3–5·4	No biopsy	—
—Mey.	28–21·5	24·5→68·4	2·7–4·2	3·3–5·8	Squamous	—
+ Six patients described on Table 1 and 2.						
		Normal response — (eighteen out of thirty-two patients)				
ranges:	7·8–28	9–32→40–60	2–8·1	0·5–15·7	5 oat cell	
means:	17·7	18→47·8	4·3	4·9	10 differentiated 3 no biopsy	4+ 14—

mequiv/l and urinary sodium was low (4–10 mequiv/day) [10].

All these symptoms were corrected by the administration of cortisone. On admission plasma corticoids were very low but the urinary excretion of 17-hydroxycorticosteroids was still normal. The response to ACTH was insufficient but obviously present. There was no response to methopyrone. The PBI was normal but no gonadotrophins were detected in urine. The radiography of the sella turcica showed a destruction of the posterior clinoids and there was a bitemporal hemianopsia. At autopsy, the pituitary was invaded by a retrochiasmatic metastase; the adrenals were largely invaded, weighing respectively 44 and 41 g with little normal tissue left, but in view of the response to ACTH, the hypocorticism of this patient must be considered as of pituitary origin [8].

In no patient was the excretion of 17-hydroxycorticosteroids two standard deviations below the mean control figure. The frequency of low 17-ketosteroid excretion (mean: 4·8 mg per day from 103 measurements in fifty-nine patients) was striking but the age and the generally poor condition of these patients must be taken into account.

Response to ACTH: in four patients, the response to ACTH was insufficient (Table 4). By all other clinical and biochemical criteria the adrenals of these patients were normal.

DISCUSSION

Our results show that adrenal hyperactivity is not uncommon in bronchial carcinoma and this confirms the observations of Hymes and Doe [11], Werk *et al.* [12], Belsky and Marles [13]. Increased plasma corticosteroids and/or excessive corticosteroid excretion were found at one time or another in 20 per cent of our patients. This was observed in differentiated as well as in oat cell carcinoma. Of the sixteen patients presenting suggestive evidence of excessive corticol production, eight had an oat cell carcinoma and eight some form of differentiated tumour. The "hypercorticism" was more related to the advanced stage of the disease than to the histology of the neoplasm and eleven of these sixteen patients died within 3 months of observation. The hypercorticism was always moderate. Hypokalemic alkalosis or gross diabetes were never observed. Therefore, these observations are quite different from what is seen in the cases described as paraneoplastic Cushing's syndromes in which the histologic pattern is practically always oat cell or anaplastic carcinoma, biologic disturbances are quite severe, and cortisol production is most often extremely high [1, 2, 4]. The production of hydrocortisone in the paraneoplastic endocrinopathies is commonly superior to 100 mg and even 200 mg [14] a day, which corresponds to Porter and Silber chromogens in urine, well above 25–50 mg per 24 hr [2, 15]. The latter figures are thus much higher than the highest found in our series.

Moreover, in the Patients Tr. and Thi. (Table 2) with oat cell carcinomas, and high values of both plasma and urinary corticosteroids, the steroid excretions were inhibited by small doses of dexamethazone. Such an inhibition would not have been observed in the case of an autonomous production of ACTH-like material thought to be characteristic of the Cushing's syndrome of bronchial carcinoma. Cushing's syndrome associated with lung cancer appears therefore from our

observations to be a distinct entity rather than the upper extreme of a spectrum of cortical activities in bronchial carcinoma.

No case of primary adrenal insufficiency was detected in this study. The extreme rarity of adrenal insufficiency in lung carcinoma despite the frequency of adrenal metastases [16] is of interest. It can probably be explained by the considerable possibilities of compensation of the adrenal glands: when maximally stimulated they are able to hypertrophy and secrete up to 300–400 mg of cortisol per day precisely as is observed in paraneoplastic Cushing's syndromes; this represents 10 to 20 times the normal production of cortisol. The pituitary where metastases are by no means exceptional [17–19] should be much more sensitive to the latter, since it is enclosed in a bony structure which limits space for expansion; it is also particularly dependant on delicate vascular and nervous connections with the hypothalamus. This means that in a cancerous patient, in the presence of clinical hypoadrenalism, one must look carefully for pituitary invasion [20]. The case of patient Van C. (Table 3) illustrated the point very well: pituitary insufficiency developed through a small retrochiasmatic metastase; when given ACTH, plasma corticoids increased as they would in any hypopituitarism, despite the large destruction of both adrenals by voluminous metastases.

However it is possible that adrenal metastases might diminish the functional reserves of some patients. The cases of the four individuals, neither cachectic nor terminal who presented a subnormal response to ACTH should perhaps be interpreted in the light of this discussion despite the absence of any clinical evidence of either pituitary or adrenal metastatic invasion.

The possible interference of liver metastases in standard appraisal of adrenal function deserves a few words of comment. Liver failure could diminish steroid excretion and alter in various ways the response to ACTH; however its effects are difficult to separate from the influence of the stress attending generalized disease and which may act in an opposite direction. Both factors, liver metastases and stress, evolve indeed in parallel as can be seen in our series where 80 per cent of the patients with increased plasma and urinary corticosteroids (Table 2) had liver metastases against 31 per cent at most in our bronchial carcinomas with apparently normal adrenal function, However, that liver metastases might lower Porter and Silber chromogens excretion (and cortisol production provided that cortisol metabolism is not qualitatively altered) is suggested in patient Da. (Table 2): within the 9 months of observation, liver function deteriorated, plasma cortisol concentrations were stationary or increased but urinary 17-hydroxycorticosteroids fell markedly. A similar discrepancy between plasma and urinary values was observed in patient Lie. (Table 1) where the liver was also largely destroyed by metastases.

RÉSUMÉ

La fonction surrénalienne a été étudiée dans 76 *cas consécutifs d'épithélioma bronchique. Aucun cas de "syndrome de Cushing paranéoplasique" n'a été observé. La fréquence de ce syndrome est donc très faible, en dessous de* 1,3% *de tous les cancers bronchiques dans notre expérience.*

Chez 20% *des patients, nous avons constaté une excrétion élevée des* 17*-hydroxy-corticostéroïdes, associée ou non à une concentration plasmatique accrue. Chez un tiers des malades, le test à l'ACTH démontrait une hyperréponse des surrénales. Cependant, dans tous ces cas, l'hyperfonctionnement surrénalien était modéré et n'avait aucune relation absolue avec l'histologie du néoplasme; de plus, après administration de dexaméthazone, une inhibition rapide de l'excrétion des stéroïdes fut observée. Nous n'avons donc trouvé aucun élément qui permette de conclure à l'existence de formes frustes de syndrome de Cushing causé par des tumeurs "non endocrines."*

Un patient présentait de l'insuffisance hypophysaire par envahissement métastatique Nous n'avons observé aucun cas d'insuffisance surrénalienne primaire par destruction métastique des surrénales; néanmoins, chez 1 *malade sur* 8, *la réserve surrénalinnee était diminuée.*

SUMMARY

Adrenal function was studied in seventy-six consecutive cases of bronchial carcinomas and no single case of "paraneoplastic Cushing's syndrome" was found. This confirms the fact that Cushing's syndrome caused by "non endocrine tumours" is a rare occurrence, the

frequency of which in our experience is below 1/76 = 1·3 *per cent of all cases of bronchial carcinomas.*

Increased plasma and/or urinary 17 *hydroxycorticosteroids were observed in* 20 *per cent of the patients. Excessive response to ACTH was found in one third of the cases. However, the adrenal hyperactivity was very moderate, unrelated to the histology of the neoplasm, and when tested, easily suppressed by dexamethazone. Therefore, we did not find any evidence for the existence of subclinical variants of the Cushing's syndrome produced by "non endocrine" tumours.*

There was a case of hypopituitarism due to metastatic destruction of the hypophysis. However, primary adrenal insufficiency due to adrenal metastases was not observed despite the fact that, in four instances out of thirty-two, the ACTH test revealed a diminished functional reserve.

ZUSAMMENFASSUNG

Die Nebennierenfunktion wurde in 76 *aufeinanderfolgenden Bronchienkrebsfällen studiert. Es wurde kein paraneoplasisches "Cushings Syndrom" beobachtet. Die Häufigkeit dieses Syndroms ist also sehr gering; es wurde in weniger als* 1,3% *aller studierten Bronchienkrebse beobachtet.*

Bei 20% *der Patienten wurden eine erhöhte Plasmakonzentration und/oder eine große Ausscheidung der* 17 *Hydroxycorticosteroiden festgestellt. Der ACTH-Test zeigte bei einem Drittel der Patienten eine Nebennierenübertätigkeit. Diese war jedoch in allen Fällen gemässigt und hatte keine Verbindung mit der Gewebsstruktur des Neoplasmas; ferner wurde nach der Einnahme von Dexomethazon eine schnelle Hemmung der Steroidaussonderung beobachtet. Wir haben also kein Element gefunden, das zu dem Schluß bringt, daß durch "nicht-endokrine" Tumore veranlaßte unbestimmte Formen des "Cushings Syndrome" bestehen.*

Ein Patient zeigte eine durch Bildung von Metastasen entstandene Hypophysen· insuffizienz. Keinen Fall von primärer Nebenniereninsuffizienz wurde durch eine metastatische Zerstörung der Nebennieren beobachtet. Jedoch hatte die Nebennierenreserve bei jedem achten Kranken abgenommen.

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The Mechanism of the Dying Off of Tumour Cells as a Result of Radiotherapy

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INTRODUCTION

THE THEORY to be developed in this paper is based on the assumption that in a given type of mitotically active cells the application of a radiation dose of 1 rad results in a specific chance ϵ of any of the daughter cells to die off.

First of all some formulae will be obtained by which, in certain cases, the value of ϵ can be derived from empirical data. In an application of these formulae to the basal cells of epidermal tumours two conclusions will be obtained which are likely to agree with radiotherapeutic evidence.

Next a hypothesis will be presented on the mechanism by which mitotically active cells die off in radiotherapy. This hypothesis will be considered in connection with the values of ϵ obtained, and it will then be shown to result in quantitative suggestions on RNA formation in the basal cells of normal epidermis and of epidermal tumours.

1. *Fundamental formulae*

(*a*) We first consider a set of N_0 similar living units which, like the basal cells of the skin, produce non dividing new units at regular intervals. We imagine that this set is subjected to a radiation dose of q rad and we will assume that any dividing unit to which a dose of 1 rad is applied has a chance ϵ to die off. The number N_q of surviving units may then be expected to be

$$N_q = N_0 e^{-q\epsilon} \tag{1}$$

and the probability λ that none of them will survive can be calculated to be

$$\lambda = \{1-(1-\epsilon)^q\}^{N_0} \tag{2}$$

The formulae (1) and (2) can be deduced as follows.

As long as no radiation is applied the number N_0 of dividing units will not change. Application of a radiation dose q, however, will reduce this number to N_q. If this dose is increased by a quantity dq the number N_q will be further reduced by an amount $-dN_q = \epsilon N_q\, dq$ and this equation is solved by formula (1).

The probability of a given dividing unit to survive the application of 1 rad is $1-\epsilon$ and so the probability that it will survive the application of q rad will be $(1-\epsilon)^q$.

The probability that it will not survive the latter application is therefore $1-(1-\epsilon)^q$ and the probability that none of the N_0 units will survive is given by the formula (2):

$$\lambda = \{1-(1-\epsilon)^q\}^{N_0}$$

(*b*) Next we consider a set of N_0 units, each of which divides in two similar units when its natural lifetime T has elapsed. T is then the doubling time of the set and after a part t of the first doubling time the number N_t of units will be

$$N_t = N_0\left(1+\frac{t}{T}\right) \tag{3}$$

Now we imagine that in the time t a radiation dose of q rad is applied to the set. The

probability λ that none of its units or of their descendants will survive is then given by the formula

$$\lambda=\{1-(1-\epsilon)^q\}^{N_0\left\{1+\frac{t}{T}\left(1-\frac{1-(1-\epsilon)^q}{\epsilon q}\right)\right\}} \quad (4)$$

In our applications the fraction

$$\frac{1-(1-\epsilon)^q}{\epsilon q}$$

will be so small that it may be neglected. By this neglection, formula (4) is transformed into

$$\lambda=\{1-(1-\epsilon)^q\}^{N_0\left(1+\frac{t}{T}\right)} \quad (5)$$

Formula (4) can be deduced as follows. If no radiation is applied, $N_0\,t/T$ of the original units will divide in the time t and $N_0(T-t)/T$ in the remaining part of the first doubling time. In fact the latter units are all subjected to a dose of q rad, and the probability λ_2 that none of them will survive is

$$\lambda_2=\{1-(1-\epsilon)^q\}^{N_0\frac{T-t}{T}} \quad (4a)$$

In order to calculate the probability λ_1 that all offspring of the former units will die off we consider the $N_0\Delta t/T$ of them which should divide in the time between t and $t+\Delta t$. These units are exposed to a radiation dose $q\tau/t$, and so the chance of any of them to dying off will be

$$1-(1-\epsilon)^{q\frac{\tau}{t}}$$

and the chance to survive

$$(1-\epsilon)^{q\frac{\tau}{t}}$$

In case of survival it will divide and both its direct descendants will then be subjected to a radiation dose of $q(t-\tau)/t$ rad. Consequently any of them will have a chance

$$1-(1-\epsilon)^{q\frac{t-\tau}{t}}$$

to die off. The probability of any of the $N_0\,\Delta t/T$ units, which where to divide in the time t, to have no surviving descendants should therefore be

$$1-(1-\epsilon)^{q\frac{\tau}{t}}+(1-\epsilon)^{q\frac{\tau}{t}}\left\{1-(1-\epsilon)^{q\frac{t-\tau}{t}}\right\}^2=$$

$$=1-2\,(1-\epsilon)^q+(1-\epsilon)^{2q}\,(1-\epsilon)^{-q\frac{\tau}{t}}$$

and so the probability that none of them will produce a surviving descendant may be written as

$$\left\{1-2\,(1-\epsilon)^q+(1-\epsilon)^{2q}\,(1-\epsilon)^{-q\frac{\tau}{t}}\right\}^{N_0\frac{\Delta\tau}{T}}$$

Consequently we shall have

$$\lambda_1=\prod_{\tau=0}^{\tau=t}\left\{1-2\,(1-\epsilon)^q+(1-\epsilon)^{2q}\,(1-\epsilon)^{-q\frac{\tau}{t}}\right\}^{N_0\frac{\Delta\tau}{T}} \quad (4b)$$

In case of small values of ϵ ($<10^{-2}$) and large values of q ($>10^3$) this formula can be transformed as follows:

$$\ln\lambda_1=\sum_{\tau=0}^{\tau=t}N_0\frac{\Delta\tau}{T}\left\{-2\,(1-\epsilon)^q+(1-\epsilon)^{2q}\,(1-\epsilon)^{-q\frac{\tau}{t}}\right\}=$$

$$=\frac{N_0}{T}\int_0^t d\tau\left\{-2\,(1-\epsilon)^q+(1-\epsilon)^{2q}\,(1-\epsilon)^{-q\frac{\tau}{t}}\right\}=$$

$$=\frac{N_0}{T}\left\{-2t\,(1-\epsilon)^q-(1-\epsilon)^{2q}\,t\,\frac{(1-\epsilon)^q-1}{q\ln(1-\epsilon)}\right\}=$$

$$=\frac{N_0}{T}(1-\epsilon)^q\,t\left\{-2-\frac{1-(1-\epsilon)^q}{q\ln(1-\epsilon)}\right\}$$

Hence

$$\ln\lambda_1=-N_0\,(1-\epsilon)^q\,\frac{t}{T}\left(2-\frac{1-(1-\epsilon)^q}{\epsilon q}\right)$$

whilst according to (5a)

$$\ln\lambda_2=-N_0\,(1-\epsilon)^q\,\frac{T-t}{T}$$

As $$\lambda=\lambda_1\lambda_2 \quad (4c)$$

and consequently $\ln\lambda=\ln\lambda_1+\ln\lambda_2$ we may conclude that

$$\ln\lambda=-N_0\,(1-\epsilon)^q\left\{1+\frac{t}{T}\left(1-\frac{1-(1-\epsilon)^q}{\epsilon q}\right)\right\}$$

and this equation is equivalent to (4):

$$\lambda=\{1-(1-\epsilon)^q\}^{N_0\left\{1+\frac{t}{T}\left(1-\frac{1-(1-\epsilon)^q}{\epsilon q}\right)\right\}}$$

In certain cases the formulae (1) and (5) can help us to derive the values of ϵ from empirical data; in the following section we will exemplify this by applying them to the basal cells of normal epidermis and of epidermal tumours.

2. *Application of the formula to the basal cells of normal epidermis and of epidermoid tumours*

(*a*) *Normal Epidermis.* The application of a radiation dose of 5000 rad to normal human skin in about 4 weeks usually results in extensive epidermolysis. It is generally assumed that this phenomenon is ultimately due to radiation death of basal cells. Recovery of the denudated area then originates both at the borders and at islets in the central parts, and these islets are likely to derive from basal cells that have survived. According to formula (1) the fraction of surviving cells will be

$$\frac{N_{5000}}{N_0}=\exp(5000\,\epsilon)$$

and if this fraction is assumed to be between 0·002 and 0·0005 we shall have

$$0{\cdot}002>\exp(5000\,\epsilon)>0{\cdot}0005$$

and, consequently

$$1{\cdot}2\times10^{-3}<\epsilon<1{\cdot}5\times10^{-3} \quad (6)$$

(*b*) *Epidermal tumours.* As basal cell carcinomas are more radiosensitive than basal cells of normal epidermis we may expect that their value of ϵ will be larger. An estimate of this value can be obtained with the help of formula (5). We will apply this formula to tumours of 0·5–1 g. The number of cells in tumours of this size is of the order of 10^8, and we will, therefore, put $N_0=10^8$. We know that 90 per cent of these tumours can be cured by the application of a radiation dose of 5000 rad in about 20 days, and as cure of a tumour implies that none of its cells survive we may put $\lambda=0{\cdot}9$

and $q=5000$. Moreover, autoradiographs of normal epidermis and of epidermoid tumours obtained after injection of tritiated thymidine suggest that the doubling time T of basal cells is about 100 days in both cases (Johnson a.o. p. 460) and so it follows from formula (5) that

$$0{\cdot}9=\{1-(1-\epsilon)^{5000}\}^{10^6\times 1{\cdot}2}$$

and hence

$$\epsilon=4{\cdot}2\times 10^{-3} \qquad (7)$$

It should be observed that this result does not markedly depend on the values assumed for N_0 and T. By substitution in (5) we obtain

$$\lambda=\{1-(1-4{\cdot}2\times 10^{-3})^{q}\}^{N_0\left(1+\frac{t}{T}\right)} \qquad (8)$$

or approximately

$$\lambda=(1-\exp(-4{\cdot}2\times 10^{-3}q))^{N_0\left(1+\frac{t}{T}\right)} \qquad (8a)$$

According to this formula the chance of cure λ of an epidermal tumour strongly depends on the radiation dose applied (q) and on the size of the tumour (N_0) and only slightly on the overall time of application (t). The curves in Fig. 1 show the relations between λ and q which correspond to different values of $N_0\,(1+t/T)$; viz. to tumours of about 0·8 cm³ $\{N_0\,(1+t/T)=10^8\}$, 0·8 mm³ $\{N_0\,(1+t/T)=10^5\}$ or 0·0008 mm³ $\{N_0\,(1+t/T)=10^2\}$

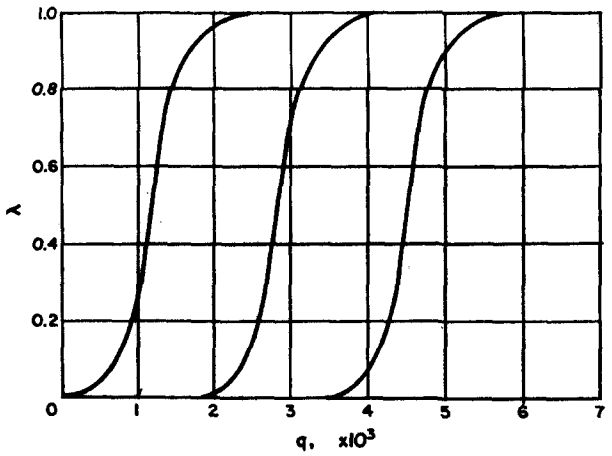

Fig. 1. *Chances* λ *to obtain a cure by the application of doses of q rad to epidermal tumours of different sizes (0.0008 mm³. 0.8 mm³ and 0.8 cm³)*

These curves clearly demonstrate the well known fact that the dose required to obtain a reasonable chance of a cure in a given tumour is rather sharply defined. Apart from that, they suggest that the dose which results in a chance of a cure of 90 per cent is rather smaller for small tumours than for larger ones. This conclusion too, though it has hardly been explicitly derived from observations, is likely to be in accordance with experience and as it could justify the application of rather low doses in postoperative radiotherapy it may even be of considerable practical importance.

Formula (8a) can be transformed into

$$q=550\log\frac{N_0\left(1+\frac{t}{T}\right)}{-\ln\lambda} \qquad (8b)$$

In this form it gives the dose q required to obtain a given chance λ of a cure by treatment of a tumour of N_0 cells in a given overall time t. In Fig. 2 are shown the relations between q and $N_0\,(1+t/T)$ which correspond to the values of λ of 0·9 and 0·7.

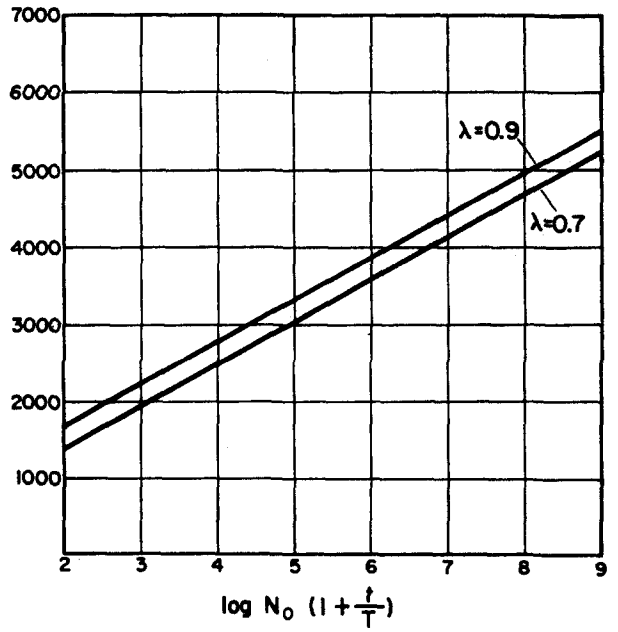

Fig. 2. *Doses q required to obtain chances of a cure* $\lambda=0{\cdot}9$ *and* $\lambda=0{\cdot}7$ *when applied in given times t to tumours of different sizes* (N_0 *cells*). *T is doubling time of tumour cells.*

3. *A theory on the mechanism of dying off of tumour cells in radiotherapy.*

In curative radiotherapy of a malignant tumour both the tumour and the surrounding normal tissues are subjected to a radiation dose of the order of 5000 rad. The aim of this procedure is that the tumour cells will die off, while no important changes will occur in the normal tissues. The sort of damage brought about in the latter tissues should therefore not be drastic, and so we may expect that the changes which result in the death of the tumour cells will not be extensive either. Now the mildest type of radiation induced permanent changes is the one which causes mutations, and as mutations may make cells unviable we will consider the possibility that the death of tumour cells caused by radiotherapy is due to the induction of unviable mutations. Cell mutations are generally considered to result from modi-

fications in the sequences of purine- and pyrimidine bases along the strands of DNA-molecules. The structural pattern of thes strands is shown in Fig. 3. In a DNA-molecule

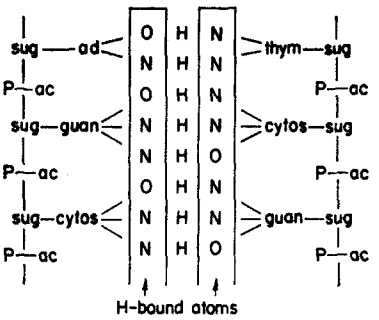

Fig. 3. Structural pattern of DNA molecules.
sug = sugar-molecule.
P. ac = molecule of phosphoric acid.
ad = adenine
thym = thymine
guan = guanine
cytos = cytosine
} *molecule*
Two long strands are connected by hydrogen bonds between N- and O-atoms.

two of them are coiled around each other to form a double helix. Their connection is brought about by hydrogen bonds between either adenine and thymine or cytosine and guanine. The sequences of these purine- and pyrimidine bases are, therefore complementary in the two strands and both of them are characteristic of the DNA molecule in question. There are two hydrogen bonds between adenine and thymine and three between cytosine and guanine, the former being of the types —O—H—N= and =N—H—N= and the latter of the types —O—H—N=, =N—H—=N and =N—H—O—. (Perutz, p. 65). The characteristic sequences of the four bases in the two strand of a DNA molecule are transmitted from a cell to its daughter cells by a special mechanism: Prior to mitosis any cell passes through a period in which the different parts of its DNA strands are successively uncoiled and separated, and then every strand gradually completed to a full DNA molecule by gradual building up of a new complementary strand. Every DNA molecule of a cell is thus replaced by two identical copies, one for every daughter cell, and so the transmission of their characteristic structure to these daughter cells is secured.

In the mechanism of copying described, coiling and hydrogen bonding appear as the essential features in the conservation of the characteristic sequences of the purine- and pyrimidine bases in the strands of DNA-molecules. We may therefore expect that changes in these sequences will arise by preference in parts of the molecules where the composing strands are uncoiled and separated, such as occurs in the periods of DNA synthesis and of RNA-formation. In these periods the atoms whose state is particularly affected are the hydrogen-unbound N- and O atoms; changes induced in these periods by ionizing radiations are, therefore, most likely to be due to the detachment of electrons from these particular atoms. It seems indeed improbable that other atoms — for instance those of the purine- or pyrimidine cycles — should be affected by preference in the premitotic phase and rather in tumour cells than in normal cells of a similar type. Consequently, we will consider the N- and O electrons mentioned as targets which in the course of the lifetime T of a cell are radio-sensitive during the period P_0 of DNA synthesis and the period (s) P_1 ($P_2 \ldots .$) of local RNA formation. Detachment of a target-electron from a DNA strand in such a period should, in this line of thought, result in non-development or unviability of the corresponding daughter-cell. An estimate of the length of P_0 can be derived from the duration of the S phase in which DNA synthesis occurs in a cell. Autoradiographs obtained after injection of tritiated thymidine suggest that this phase lasts about 6 hr (Johnson a.o., p. 460, Devik, p. 63). We may expect that in any given cell this is a period of simultaneous radiosensitivity of all its targets. Now in consequence of this concentration of sensitivity in a short space of time the DNA periods of particular cell-radiosensitivity will be rather unimportant in radiotherapy. It is indeed unlikely, that in a tumour irradiated in short daily sessions for some 4 to 8 weeks a target electron will be detached in such a period in a major part of its cells, and the same holds for a tumour which is treated continuously — by puncture or by moulds — during a week or less. Cure of a tumour by these current procedures can therefore in the context of the given theory, hardly be due to detachment of target electrons in DNA periods of radiosensitivity and should rather result from the detachment of such electrons in their RNA periods which are distributed at random over the lifetime of a cell.

In the following section the quantitative aspects of this concept will be considered in connection with the values of ϵ derived in §2 from empirical data.

4. *Quantitative aspects of the theory*

We will consider a cell with lifetime T in which P is the mean effective time of radiosensitivity of its target electrons. Let ω be the

probability that the application of 1 rad to this cell will release one or more target electrons from a particular DNA strand. According to our theory such an application will then have a probability $\omega P/T$ to result in nondevelopment or unviability of the daughter cell which corresponds to the strand in question. In §1 this sort of probability has been denoted by ϵ, and so we have

$$\epsilon = \omega \frac{P}{T}$$

This formula will now be applied to the basal cells considered in §2.

As to the lifetime T of these basal cells we have seen that $T = 100$ days may be considered a reasonable estimate.

With regard to the value of P we have arrived at the view that the effective time of radiosensitivity of a target-electron consists of the periods in which its N- or O atom is involved in RNA formation. We will now assume that these periods have about the same duration as the DNA periods, i.e. approximately 6 hr or $\frac{1}{4}$ day. If their mean number per target is denoted by p we may, therefore, put $P = p/4$ days.

Lastly we will consider the quantity ω. In an analysis of this quantity we will make use of the following numerical data:

1. number of electrons per gramme tissue: $3{\cdot}3 \times 10^{23}$ (Johns, pp. 695, 696, 703).
2. number of these electrons released by 1 rad: $1{\cdot}8 \times 10^{12}$ (Johns, pp. 14, 195, 651).
3. number of base pairs in DNA of a human cell: 10^9 (Perutz, p. 63).
4. number of hydrogen-bound N- or O atoms per base in DNA: 2 or 3 (Perutz, p. 65).
5. number of electrons per N- or O-atom: 10 (2 in the *K*-shell and 8 in the *L* shell).

From 3, 4 and 5 it follows that the number of electrons which in the DNA-molecules of a human cell belong to hydrogen-bound N- and O-atoms is approximately $(2{\cdot}5 \pm 0{\cdot}5) \times 10^{11}$ per daughter cell. A fraction of these electrons has been supposed to be the targets of the cell, and if this fraction is denoted by η the number of targets will be $(2{\cdot}5 \pm 0{\cdot}5) \times 10^{10} \times \eta$ where $\eta \leqslant 1$.

In view of 1 and 2 the probability ω that on application of 1 rad at least one electron of this set will be detached from its atom then amounts to

$$\omega = \frac{1{\cdot}8 \times 10^{12}}{3{\cdot}3 \times 10^{23}} \times (2{\cdot}5 \pm 0{\cdot}5) \times 10^{10} \eta = \\ = (13{\cdot}5 \pm 2{\cdot}7) \times 10^{-2} \eta$$

Hence

$$\epsilon = \omega \frac{P}{T} = (13{\cdot}5 \pm 2{\cdot}7) \times 10^{-2} \eta \times \frac{0{\cdot}25 p}{100} = \\ = (0{\cdot}34 \pm 0{\cdot}07) \times 10^{-3} \eta p \qquad (10)$$

This result will now be considered in connection with the values of ϵ obtained in §2 for basal cells of normal epidermis and of epidermoid tumours.

It follows from (6) and (10) that in the basal cells of normal epidermis $\eta p \sim 4$. Hence, as $\eta \leqslant 1$, the mean number p of RNA periods of a target electron should not be less than 4 and its mean effective time P of radiosensitivity not less than 1 day. Now there is no conclusive evidence why the number p should be considerably larger than 4. In fact, a great many targets serves the production of enzymes which, perhaps, are scarcely subject to consumption and replacement, and consequently not necessarily needed more than once in the lifetime of a cell. On the other hand η should be expected to be smaller than 1, for in cells with a certain degree of differentiation part of the hydrogen-bound N- or O atoms of DNA molecules will have no vital functions, and replacement of the corresponding purine- and pyrimidine bases by others will therefore not affect the viability of the pertaining daughter cells. Hence it seems reasonable to estimate η between 0·8 and 0·2 and, consequently, p between 5 and 25. The corresponding numbers of target electrons per daughter cell will then be something between 2×10^{10} and 4×10^9.

We may expect that in the basal cells of epidermal tumours the value of η and the number of target-electrons will be about the same as in those of normal epidermis. The mean number p of RNA periods per target will then be larger. It follows from (7) and (10) that in the basal cells of epidermoid tumours $p = 12$ to 16. Consequently p will be 3 to 4 times as large as in normal cells and have a value between 15 and 80. In Table 1 are given the values of p and P which in normal epidermis and in epidermal tumours correspond to different values of η.

The values of P and p given in Table 1 correspond to a doubling time T of 100 days. However it must be mentioned that several authors have given lower estimates like 50 or even 20 days (Montagna, p. 70), and such estimates result in values of p and P which are 1/2 to 1/5 of those of Table 1. The given values are therefore to be accepted with reservation and should be revised when more precise information on the doubling time T will be available.

Table 1

T= 100 days	basal cells of normal epidermis		epidermal tumours	
η	p	P	p	P
0·8	5	1 day	18	4·5 days
0·6	7·5	2 days	24	6 days
0·4	10	2·5 days	36	9 days
0·2	20	5 days	72	18 days

T=doubling time of cells.
η=target fraction of electrons of hydrogen bound N- and O-atoms in DNA-molecules.
p=mean effective number of RNA-periods of radiosensitivity per target electron.
P=mean effective time of RNA-radiosensitivity per target electron.

On the whole, however, Table 1 suggests that in tumour cells there is an excess of RNA-production. This suggestion is well in line with the approved view that the increase of metabolic activity of tumour cells is due to defects in inhibitory mechanisms.

RÉSUMÉ

La théorie présentée dans cette communication est fondée sur l'hypothèse que, dans un type donné de cellules à activité mitotique, l'application d'une dose de 1 *rad aboutit à une probabilité spécifique* ϵ *pour chaque cellule-fille de perdre sa viabilité.*

On a d'abord obtenu quelques formules mathématiques qui, dans cetrains cas, permettent de déduire les valeurs de ϵ *à partir de données empiriques. Ces données sont appliquées aux cellules basales de l'épiderme normal et de tumeurs épidermiques. Pour ces tumeurs, on aboutit aux conclusions suivantes qui sont apparemment confirmées par l'expérience:*

1. *La dose nécessaire pour arriver, par radiothérapie, dans un temps donné, à une probabilité raisonnable de guérison d'une tumeur donnée est assez précisément définie.*
2. *Cette dose dépend du volume de la tumeur.*

Ensuite, les valeurs de ϵ *obtenues pour les cellules basales de l'épiderme normal et de tumeurs ont été considérées en relation avec une hypothèse sur le mécanisme qui, en radiothérapie, rend non viables les cellules à activité mitotique. Les points principaux de cette hypothèse sont les suivants:*

(*a*) *Dans une cellule ayant une activité mitotique, une partie des électrons des atomes N et O, à liaison hydrogène, dans les chaînes d'ADN, sont des cibles.*

(*b*) *Un électron-cible est radiosensible quand la liaison hydrogène de son atome a été rompue, c'est-à-dire dans les périodes ou cet atome est engagé dans la synthèse d'ADN ou dans la formation d'ARN.*

(*c*) *Chaque fois qu'un électron-cible est détaché de son atome, pendant une période de radiosensibilité, la cellule-fille correspondant à la chaîne ADN de cet atome ne sera pas viable.*

Un développement quantitatif de cette hypothèse conduit aux conclusions suivantes sur les cellules basales considérées dans cet article:

(*A*) *Une fraction importante, soit environ* 5/10 *des électrons des atomes N et O, à liaison hydrogène, dans les chaînes ADN, sont des cibles; par conséquent, le nombre d'électrons-cibles, par cellule-fille, est de l'orde de* 10^{10}.

(*B*) *Dans une cellule donnée, le nombre de périodes pendant lesquelles un certain atome N ou O, à liaison hydrogène, dans une chaîne ADN, est engagé dans la synthèse d'ARN, est assez petit dans l'épiderme normal* (*de* 5 *à* 20), *et sensiblement plus grand dans les tumeurs épidermiques* (*de* 20 *à* 70).

SUMMARY

The theory put forward in this paper is based on the assumption that in a given type of mitotically active cells the application of a radiation dose of 1 *rad results in a specific chance* ϵ *of any of the daughter cells to die off. This assumption will be shown to imply:*

1. *that the dose required to obtain a reasonable chance of a cure by radiotherapy of a given tumour in a given overall time is rather sharply defined and*
2. *that it depends on the size of the tumour.*

Both conclusions are likely to agree with facts.

In certain cases, e.g. in the basal cells of normal epidermis and of epidermal tumours, the values of ϵ *can be derived from empirical data. These values will be considered in connection with a particular hypothesis on the mechanism by which mitotically active cells die off in radiotherapy.*

The main points of this hypothesis are as follows:

(*a*) *In a mitotic active cell the electrons of part of the hydrogen bound N- and O atoms of the DNA-strands are targets.*

(*b*) *A target-electron is radiosensitive when the hydrogen-bound of its atom has been released, that is in the periods of involvement of this N- or O atom in DNA synthesis or in RNA formation.*

(*c*) *When a target electron is detached from its atom in a radiosensititive period, the daughter cell corresponding to the DNA strand of this atom will not develop or be unviable.*

A quantitative development of this hypothesis will be shown to suggest the following conclusions on the basal cells considered:

(*A*) *A considerable fraction, perhaps about* 0·5, *of the electrons of hydrogen-bound N- or O atoms in the strand of DNA molecules are targets; the number of target-electrons per daughter cell is therefore of the order of* 10^{10}.

(*B*) *In a given cell the mean number of periods in which a hydrogen bound N- or O atom of a DNA strand is involved in RNA formation is comparatively small —*5 *to* 20*— in normal epidermis and rather larger —*20 *to* 70*— in epidermal tumours.*

ZUSAMMENFASSUNG

Ausgangspunkt der in dieser Arbeit entwickelten Theorie ist die Annahme dass nach Bestrahlung einer bestimmten Typus mitotisch aktiver Zellart mit einer Dosis von 1 *rad jede zu entstehende Tochterzelle eine Chance* ϵ *hat sich nicht zu einer lebensfähigen Einheit zu entwickeln.*

Zuerst werden einige Formeln gegeben mit deren Hilfe sich der Wert von ϵ *in bestimmten Fällen aus empirischen Daten ableiten lässt. Diese Formeln werden dann an Hand der Basalzellen der normalen Epidermis und epidermaler Tumoren angewandt. Für epidermale Tumoren ergeben sich dabei folgende Schlüsse die anscheinend durch die Erfahrung bestätigt werden:*

1. *Bei der Strahlenbehandlung eines gegebenen Tumors zu einer gegebener Zeit ist die für eine reelle Genesungschance benötigte Dosis ziemlich genau definiert.*
2. *Diese Dosis ist von der Grösse des Tumors abhängig.*

Die fürdie genannten Basalzellen erhaltenen Werte von ϵ *werden sodann in Zusammenhang mit einer bestimmten Hypothese über den Absterbungsmechanismus mitotisch aktiver Zellen in der Strahlentherapie betrachtet. Es folgen hier die wichtigsten Punkte dieser Hypothese:*

(*a*) *In einer mitotisch aktiver Zelle sind die Elektronen der Wasserstoff-gebundenen N- und O-Atome der DNS-Ketten zum Teil "Targets."*

(*b*) *Ein Target-Elektron ist strahlempfindlich wenn sich die Wasserstoffbindung seines Atoms gelöst hat, das heisst in den Perioden in denn dieses N- oder O-Atom an der DNS-Synthese oder an der RNS-Bildung beteiligt ist.*

(*c*) *Wenn ein Target-Elektron in einer Strahlemfindlichen Periode von seinem Atom losgelöst worden ist wird sich die der DNS-Kette dieses Atoms entsprechende Tochterzelle nicht zu einer lebensfähigen Einheit entwickeln.*

Bei einer kwantitativen Entwicklung dieser Hypothese ergeben sich für die betrachteten Basalzellen folgende Schlüsse:

(*A*) *Ein grosser Bruchteil, z.B. funf Zehntel der Elektronen Wasserstoffgebundener N- oder O-Atome in den DNS-Ketten sind Targets; die Zahl dieser Elektronen pro Tochterzelle soll daher von der Ordnung von* 10^{10} *liegen.*

(*B*) *In einer gegebenen Zelle ist die mittlere Zahl von Perioden während deren ein Wasserstoffgebundenes N- oder O-Atom einer DNS-Kette bei der RNS-Bildung beteiligt ist verhältnismässig klein —* 5 *bis* 20 *— in der normalen Epidermis und merklich grösser —* 20 *bis* 70 *— in epidermalen Tumoren.*

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Comparaison des Effets Obtenus sur les Chromosomes de Cellules Sanguines Humaines Irradiées *in vitro* au moyen des Rayons X, des Rayons Gamma, du Cobalt 60 et des Electrons de Haute Energie*

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Les aberrations chromosomiques, sous l'influence des irradiations, sont connues depuis longtemps; elles ont d'abord été étudiées au niveau de structures végétales [1], puis les progrès réalisés dans les méthodes de culture tissulaire ont permis l'observation de ces aberrations sur les cellules animales [2]. Nous avons tenté, dans ce travail, de comparer les anomalies observées sur des cellules sanguines humaines sous l'influence de trois types de radiations: le rayonnement X (200 kV), le rayonnement gamma du cobalt 60, le rayonnement bêta (bétatron de 35 MeV).

METHODE [3]

(*a*) *Mise en culture*

Vingt prélèvements de sang humain appartenant à 14 femmes et 6 hommes, tous sujets normaux, ont été mis en culture selon la méthode de Moorhead et Hungerford, pendant 96 hr. Chacun de ces prélèvements a été divisé en quatre échantillons; un des échantillons a servi de témoin, les trois autres ont été irradiés, le premier par RX, le second par rayons gamma et le troisième au moyen d'électrons.

(*b*) *Irradiation des cultures*

Les cultures ont été irradiées à la 72ème heure; la température a varié entre 20 et 25°; la pression en oxygène était la pression atmosphérique. Les doses utilisées ont été de 50, 100, 200, 400 et 600 r délivrés en une séance. Afin d'être absolument sûr des doses administrées, nous avons placé, dans un flacon analogue à celui contenant la culture de sang, une petite chambre d'ionisation de Sievert. Il a été possible ainsi de déterminer avec précision la dose réellement reçue par les cultures irradiées. Les rayons X étaient émis sous 200 kV filtrés par 1 mm de cuivre; la distance source-culture était de 50 cm; le flux a varié entre 20 et 25 r/min. Le rayonnement gamma provenait d'un appareil de télécobalt de 2000 c; la distance entre la source radioactive et la culture était de 80 cm; le flux variait entre 50 et 60 r/min. Enfin, les électrons étaient émis par un bétatron de 35 MeV fonctionnant sous 20 MeV; le flux était de 50 r/min. Après irradiation, chaque échantillon a été remis en culture pour une période de 24 hr. Les cultures témoins ont également été sorties de l'étuve à la 72ème

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heure, maintenues à la température ambiante pendant toute la durée de l'irradiation des autres cultures, et repiquées pour une période de 24 hr.

(*c*) *Arrêt des cultures*[3]

Les cultures ont été arrêtées à la 96 ème heure; elles ont été colchicinisées pendant 6 hr, puis mises en milieu hypotonique, et enfin centrifugées. Le culot terminal a servi à la confection de frottis sur lames mouillées à l'eau distillée glacée; ces frottis ont été séchés à l'air et colorés 24 hr plus tard à l'orcéine.

(*d*) *Décompte des mitoses*

Pour chaque culture témoin et pour chaque culture irradiée, il a été compté 400 cellules en métaphase ou en anaphase; nous n'avons retenu que les mitoses bien étalées permettant un bon examen de tous les chromosomes. Les anomalies ont été notées par l'observation microscopique directe; seules les mitoses d'interprétation difficile ont été étudiées sur photographie. Toute image douteuse a été considérée comme normale. L'observateur a examiné chaque lame, sans connaître à quelle série elle appartenait, de manière à éviter tout élément subjectif dans l'interprétation des images.

RESULTATS

(*a*) *Dose-seuil*

L'existence d'une dose seuil revêt une importance considérable, en particulier dans les problèmes de radioprotection. Pour notre part, au cours d'un travail expérimental préalable, non publié, nous n'avons pas observé, 24 hr après l'irradiation, d'augmentation du nombre des anomalies chromosomiques avec des doses inférieures à 50 r; mais, cependant, nous ne considérons pas ceci comme une conclusion définitive, faute d'avoir examiné un nombre suffisamment grand de mitoses. Notre travail, qui consistait à comparer l'effet de divers types de radiations, nécessitait le dénombrement d'un grand nombre de mitoses anormales; des doses de radiations relativement élevées étaient donc requises.

(*b*) *Types d'altérations chromosomiques observées* (*Fig.* 1)

1. Dans les cultures-témoins, les anomalies ont été de trois types: les lésions achromatiques, les polyploïdies et les endoréduplications [4]. Sur 1600 mitoses en métaphase et en anaphase, choisies pour leur bon étalement, provenant de cultures non irradiées, nous avons noté sept lésions achromatiques (Fig. 2), quatre endoréduplications et quatre polyploïdies, soit en tout 15 anomalies sur 1600 cellules.

2. Les cultures irradiées ont montré les anomalies suivantes [5, 6];

—lésions achromatiques: nous avons considéré qu'il y avait une lésion achromatique (achromatic gap des Anglo-Saxons) chaque fois qu'il était observé une interruption au niveau d'une chromatide, sans que la longueur de celle-ci ne soit modifiée [7];

—délétion d'une chromatide ou délétion de deux chromatides au même niveau (délétion isochromatidienne) (Fig. 4);

—échange de chromatides: des échanges de chromatides entre deux ou plusieurs chromosomes différents donnent des images très facilement repérables; elles ont été principalement rencontrées dans les cultures irradiées au moyen de doses de 400 et 600 r. (Fig. 3);

—fragments de chromosomes: leur mise en évidence dans des mitoses bien étalées se fait aisément; souvent, cependant, ces fragments n'apparaissent qu'à l'examen photographi-

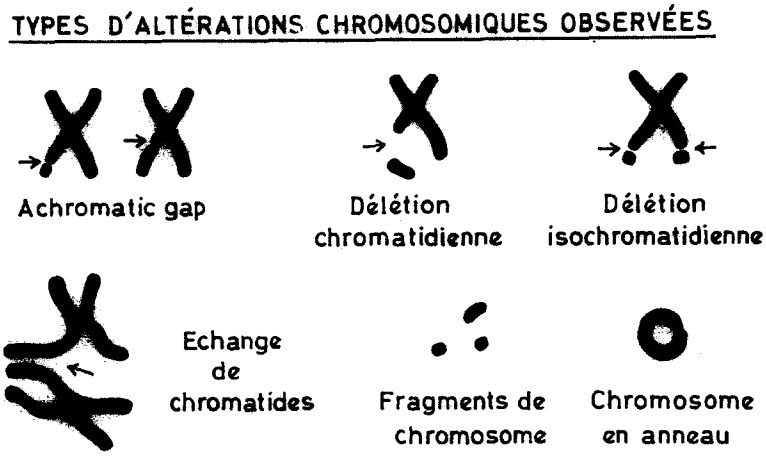

Fig. 1. *Schéma des diverses aberrations chromosomiques observées dans les cultures irradiées, à l'exception des polyploïdies et des endoréduplications.*

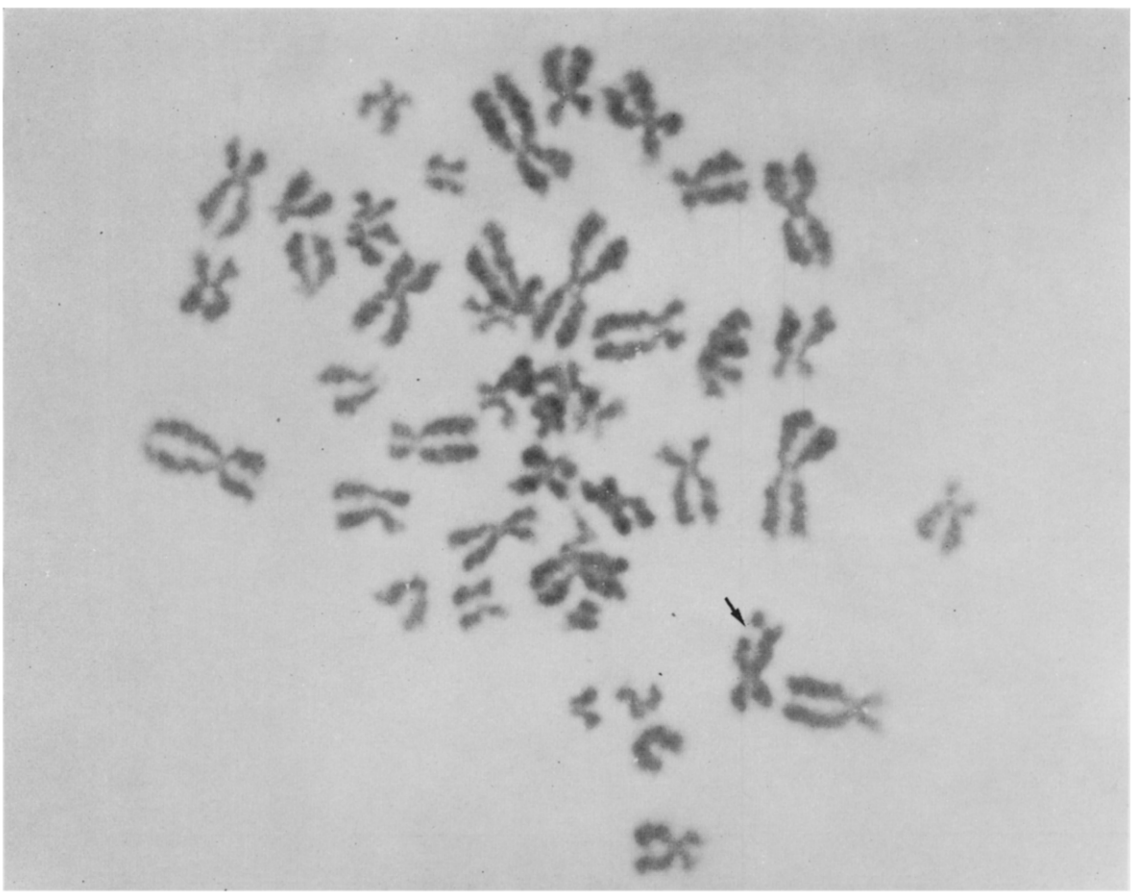

Fig. 2. Mitose provenant d'une culture-témoin non irradiée. On observe, sur un chromosome du groupe 4–5, *une lésion du type "achromatic gap".*

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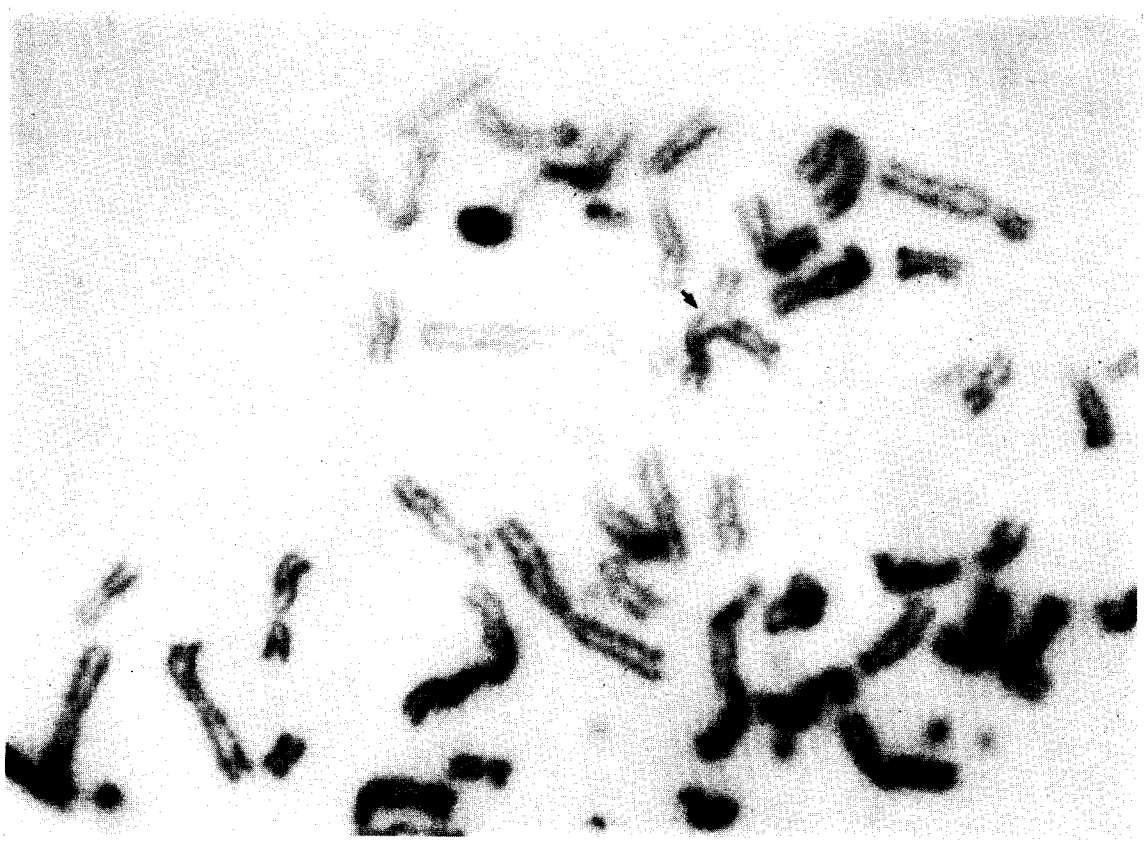

Fig. 3. *Mitose provenant d'une culture irradiée au moyen d'électrons* 20 *MeV. Dose* 200 *r. Echange typique entre chromatides de chromosomes différents.*

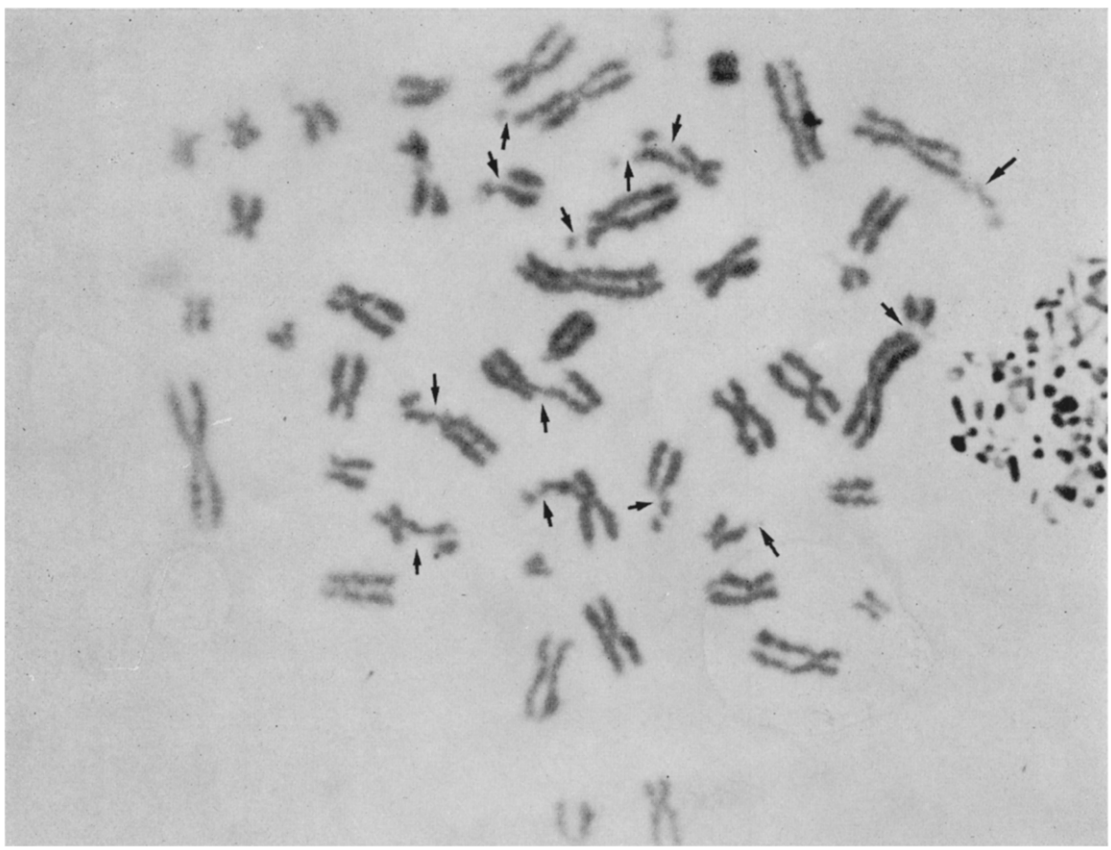

Fig. 4. Mitose provenant d'une culture irradiée au moyen de rayons gamma du Co 60. *Dose* 600 *r. Nombreux fragments, nombreuses délétions observables.*

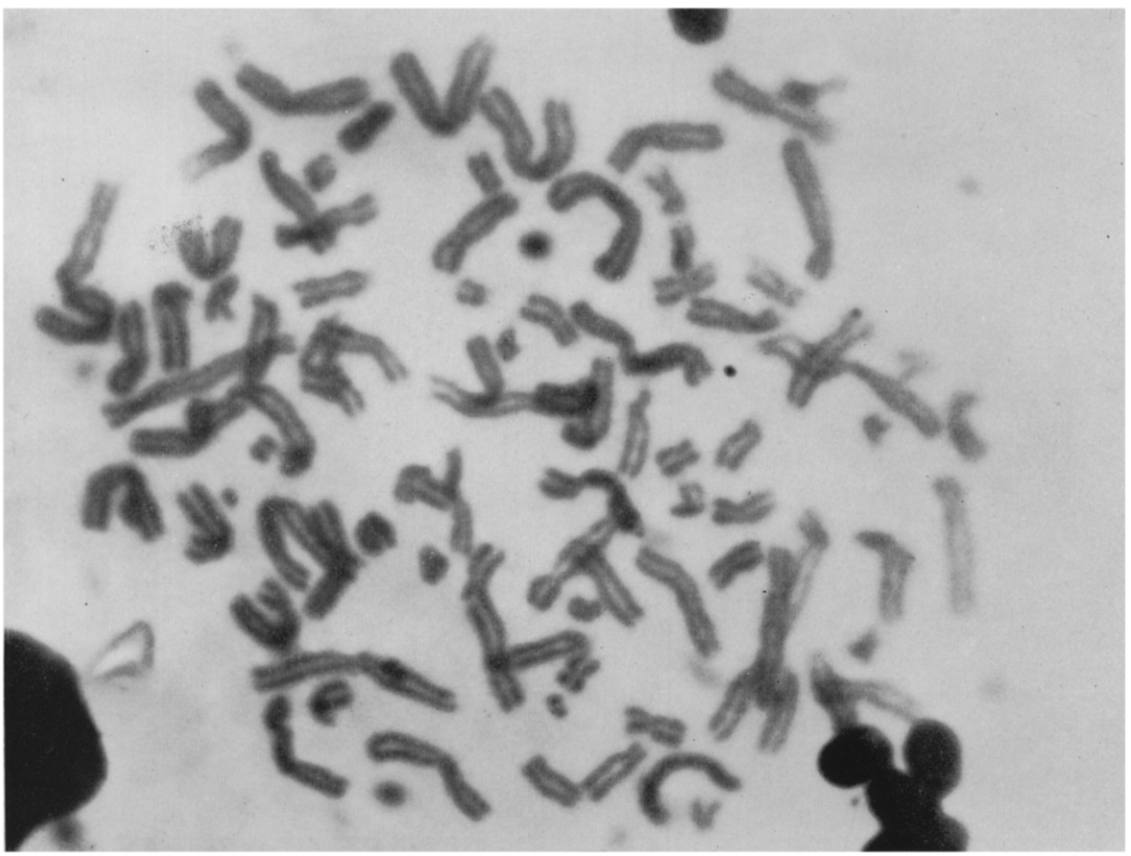

Fig. 5. *Polyploïdie, avec aberrations chromosomiques, observée dans une culture irradiée au moyen de RX.*
Dose 600 *r.*

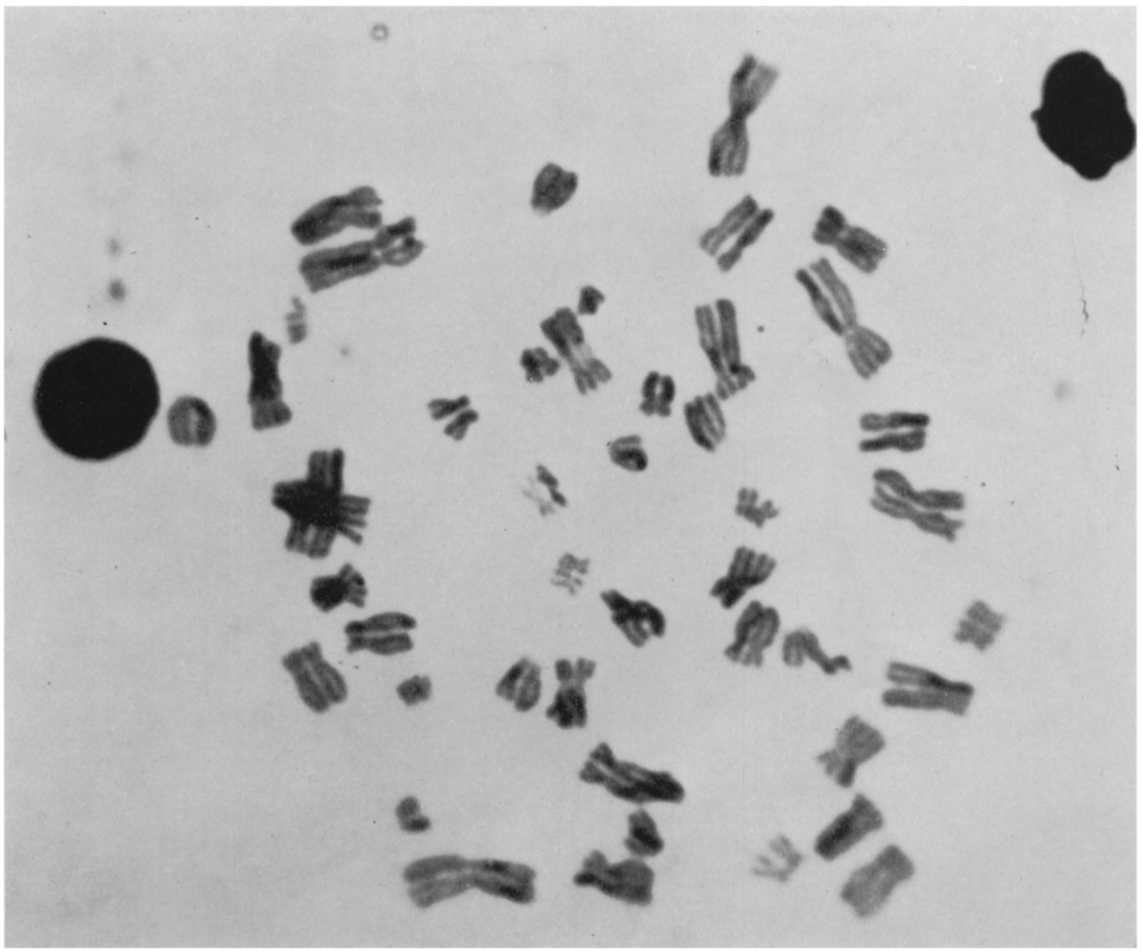

Fig. 6. *Endoréduplication, sans aberrations chromosomiques, observée dans la même culture que la Fig.* 5.

que. Il existe des fragments uniques et des fragments doubles (Fig. 4).;

—chromosome en anneau (ring chromosome): il s'agit d'un aspect facilement identifiable mais que nous n'avons rencontré que très rarement et uniquement pour des doses de 400 et 600 r;

—translocation: l'existence d'une translocation ne peut être mise en évidence habituellement que par l'étude du caryotype de chaque mitose; il s'agit là d'un travail long, et nous ne l'avons pas encore abordé. Mais, cependant, l'existence de translocations est certaine, et a été démontrée occasionnellement par l'examen de photographies de cellules irradiées. Aussi, dans notre décompte de cellules anormales, il faut considérer que le nombre d'anomalies considérées ici est inférieur à la réalité; des translocations, des inversions ont pu échapper à l'examen microscopique direct;

—chromosomes dicentriques: ce type d'anomalies n'a jamais été noté dans nos observations;

—endoréduplications (Fig. 6): le phénomène d'endoréduplication reste assez mal compris: sa fréquence dans des cultures de sang normal, examinées à la 72ème heure, est inférieure à 1 o/oo [8]; Sur 1600 cellules provenant des cultures témoins, donc non irradiées, fixées à la 96 ème heure, nous avons relevé 4 endoréduplications, soit 2,5 o/oo; il faut toutefois remarquer que le hasard a voulu que ces 4 endoréduplications ont été observées dans la même culture-témoin, correspondant au groupe irradié avec 100 r, ce qui explique le taux de 1% (4 endoréduplications sur 400 cellules étudiées) indiqué à la Fig. 7. Dans les autres cultures-témoins, nous n'avons relevé aucune endoréduplication (0/1200 cellules). Il est évident qu'il serait nécessaire d'étudier un plus grand nombre de mitoses pour tirer des conclusions valables. Nous avons trouvé un taux beaucoup plus élevé dans les cultures irradiées, le chiffre variant entre 20 et 40 o/oo [9]. Il semble y avoir augmentation de ce taux avec la dose, mais les nombres observés ne sont pas suffisamment grands pour préciser l'allure de la relation dose/effet. D'autre part, il ne paraît pas y avoir de différence entre les trois types de rayonnements utilisés (Fig. 7);

—Polyploïdies (Fig. 6): ce qui a été dit plus haut pour la fréquence des endoréduplications est valable pour les polyploïdies. Ici aussi, on admet un taux approximatif de 1 o/oo pour des cultures fixées à la 72 ème heure; nous avons observé 4 polyploïdies sur

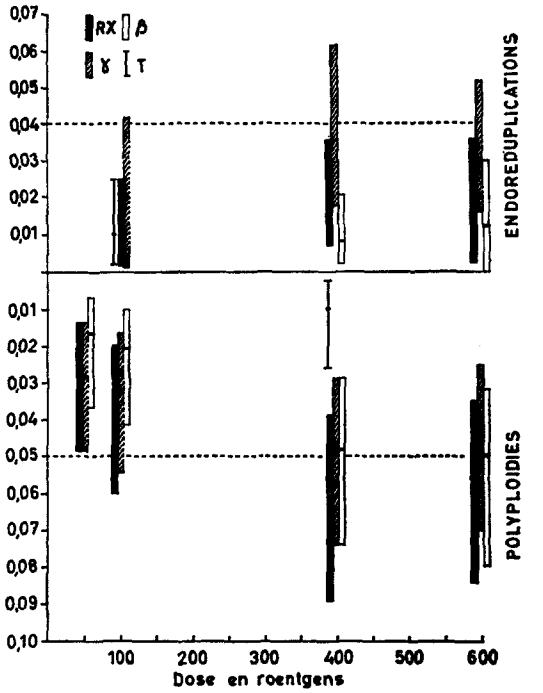

Fig. 7. Pourcentage comparatif des polyploïdies et des endoréduplications en fonction de la dose, dans les cultures irradiées avec les trois types de radiations. La hauteur des traits correspond à la variation statistique autour de la valeur observée représentée par un trait horizontal.

1600 cellules provenant de cultures-témoins fixées après 96 hr (2,5 o/oo), en fait les 4 polyploïdies ont été notées dans une même culture-témoin, celle du groupe irradié avec 400 r, les autres cultures témoins n'en présentant aucune (0/1200 cellules). Le taux des polyploïdies s'élève dans les cultures irradiées à des valeurs de 30 à 60 o/oo. Le nombre de polyploïdies semble augmenter avec la dose, les trois types de rayonnements entraînant taux semblables (Fig. 7).

(*c*) *Effet de la dose sur le nombre de cellules présentant des anomalies chromosomiques*

On peut voir, d'après le graphique (Fig. 8), que le nombre de mitoses anormales augmente avec la dose; cette augmentation pourrait être linéaire; il s'agit là d'un phénomène bien connu et d'ailleurs prévisible.

Nous insistons sur le fait que nous n'avons pas compté le nombre d'anomalies par cellule, mais bien le nombre de cellules présentant des anomalies, quelles qu'elles soient, et ce, 24 hr après irradiation.

Nous nous proposons, dans une étape ultérieure, de faire le décompte des aberrations observées dans les mitoses irradiées afin de préciser une éventuelle différence d'action des rayons X, des rayons gamma et des électrons.

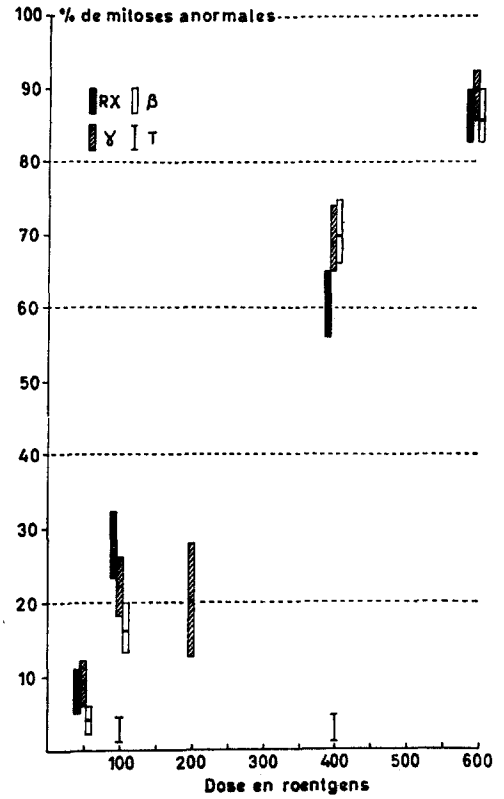

Fig. 8. Ce graphique indique, pour chacune des doses représentées en abscisse, le pourcentage de mitoses anormales observées dans les cultures irradiées au moyen des trois types de radiations. La hauteur des traits repésente la variation statistique, avec un intervalle de confiance de 95%, autour de la valeur observée représentée par un trait horizontal.

(*d*) *Effet du type des radiations utilisé*

Le même graphique met clairement en évidence qu'il n'existe pas de différence d'action des trois types de rayonnement, tout au moins en ce qui concerne le nombre de mitoses anormales notées 24 hr après l'irradiation, et pour des doses variant entre 50 et 600 r. La hauteur des traits représentés sur ce même graphique correspond à la variation statistique du nombre de cellules anormales autour de la valeur observée et cela avec un intervalle de confiance de 95%. Pour chaque dose de radiation, les résultats, pour les trois types de rayonnement utilisés, sont absolument superposables, aucune différence ne peut être notée.

Il nous manque malheureusement le décompte pour la dose de 200 r, par RX et par électrons; aucune culture irradiée dans ces conditions n'ayant permis de compter un nombre suffisamment grand de mitoses; de plus, pour la dose de 200 r par cobalt, le décompte n'a pu se faire que sur 100 cellules au lieu de 400.

DISCUSSION

A l'examen des résultats présentés, on pourrait s'étonner devant le nombre relativement levér de cellules apparemment normales alors qu'elles ont été irradiées avec des doses de 400 et 600 r. En fait, nous proposons à cela au moins trois explications: tout d'abord, il ne faut pas oublier que nous avons observé les cellules 24 hr après l'irradiation et que, étant donné ce délai, il est possible qu'un certain nombre de cellules aient été détruites à la suite d'anomalies létales; d'autre part, d'autres cellules ont pu réparer les anomalies radio-induites; il existe enfin une troisième explication que nous nous proposons de vérifier ultérieurement, et qui a déjà été évoquée plus haut: l'existence de translocations éventuelles qui, échappant à l'examen direct, ne sont décelables que par l'étude des caryotypes des cellules apparemment normales au premier examen.

Quant aux raisons qui justifient l'étude des anomalies chromosomiques 24 hr après l'irradiation, elles sont, dans une certaine mesure, d'ordre clinique. Les techniques radiothérapiques habituelles consistent à administrer des doses totales de radiations relativement élevées mais réparties dans le temps, les doses unitaires étant de l'ordre de celles que nous avons utilisées dans ce travail. Il était par conséquent intéressant, à notre avis, de noter le bilan des altérations 24 hr après l'irradiation, moment où, en thérapeutique, une nouvelle dose est administrée.

Nous pensons que la conclusion de ce travail, à savoir que les trois types de radiations utilisées ont la même action qualitative et quantitative sur les chromosomes de cellules sanguines humaines est défendable. Expérimentalement, nous nous sommes efforcés de rester dans des conditions strictement identiques: mêmes conditions de culture, mêmes doses, même température, flux d'irradiation sensiblement identique, seuls le type de rayonnement et, évidemment, son énergie ont varié. Si cette conclusion devait être confirmée par d'autres tests biologiques, il en résulterait qu'au point de vue clinique, les résultats plus favorables obtenus par les rayonnements de haute énergie (Co 60 et électrons), dans le traitement des tumeurs malignes, sont à mettre essentiellement sur le compte de leurs avantages techniques. Ces avantages sont, rappelons-le, l'irradiation moindre de la peau, permettant de délivrer des doses élevées sur un même champ cutané, le rendement en profondeur élevé, facilitant la radiothérapie des tumeurs profondes, l'absorption énergétique notablement diminuée dans le tissu osseux, écartant sensiblement le risque de nécrose, et la diminution de la dose intégrale autorisant l'administration de radiations sans altération majeure de l'état général.

Remerciements — Nous remercions ici le Dr. Koulischer, assistant au service de Cytogénétique, qui a bien voulu nous conseiller pour la mise en culture de nos échantillons de sang. Nos remerciements vont également au Dr. Hayez qui nous a assisté à examiner les nombreux documents photographiques. Enfin, nous sommes vivement reconnaissant à Mme Galperin qui s'est chargée non seulement de revoir avec nous une partie de nos photographies de mitoses mais surtout d'établir l'analyse statistique de nos documents expérimentaux.

RÉSUMÉ

Des cellules sanguines humaines, cultivées selon la méthode de Moorhead et Hungerford, ont été irradiées avec trois types de radiations : les RX 200 *kV, les rayons gamma du Co* 60 *et les électrons de* 20 *MeV. Les doses ont varié entre* 50 *röntgens et* 600 *röntgens.*

Les aberrations chromosomiques, observées 24 *hr après irradiation, sont: des lésions achromatiques, des ruptures de chromatides, des échanges chromatidiens, des chromosomes en anneau, des polyploïdies, des endoréduplications.*

Les pourcentages de mitoses anormales ont été comparés dans les cultures irradiées avec les RX, avec les rayons gamma et avec les électrons. Aucune différence statistique n'a été mise en évidence. Néanmoins, il est possible qu'une différence existe dans le nombre et dans le type des aberrations chromosomiques observées par cellule. La démonstration de ce point constitue une seconde étape, non encore achevée, dans la détermination de l'efficacité biologique relative des trois types de rayonnements.

SUMMARY

Human blood cells were cultured according to the method of Moorhead and Hungerford and irradiated with three types of radiations: X-rays of 200 *kV, gamma-rays of Co* 60, *electrons of* 20 *MeV. The doses varied between* 50 *and* 600 *r. The chromosomial aberrations observed* 24 *hr after irradiation were: achromatic gaps, chromatid breaks, chromatid exchanges, ring chromosomes, polyploidies and endoreduplications.*

The rates of abnormal mitoses were compared in the cultures irradiated with X-rays, with gamma-rays and with electrons. No statistical difference was observed. Nevertheless, it is possible that a difference exists in the number and in the chromosomial aberration types observed per cell. The demonstration of this point constitutes a second step—not yet finished—in the study of the Relative Biological Efficiency of the three types of radiations.

ZUSAMMENFASSUNG

Menschliche Blutzellen, nach der Methode von Moorhead und Hungerford bebrütet, wurden bestrahlt, u.z. der Wirkung dreier verschiedenen Strahlenarten ausgesetzt; gewöhnlicher Röntgenstrahlung (200 *kV*)*; den γ-Strahlen des* Co^{60} *und Elektronen von* 20 *MeV. Die verabreichten Dosen bewegten sich zwischen* 50 *u.* 600 *r.*

Folgende Veränderungen wurden nach 24 *Std. beobachtet: Achromasie der Chromosomen, Chromosomenbrüche, Chromosomenaustausch, Ringformelbildung, polyploide Kerne und Verklebungen.*

Die Prozentsätze der pathologischen Mitosen in den durch Rtg- und γ-Strahlen, sowie durch Elektronen bestrahlten Gewebekulturen wurden verglichen. Es konnte kein statistisch signifikanter Unterschied nachgewiesen werden. Es ist jedoch möglich, dass je nach der beobachteten Zellteilung ein Unterschied besteht, was die Anzahl und die Art der chromosomalen Veränderungen betrifft. Der Beweis dafür wird in einer noch nicht abgeschlossenen Arbeit durch die Bestimmung der relativen biologischen Wirksamkeit der drei verschiedenen Strahlenarten erbracht.

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Technical Article

Some Experiences with the Preservation of Tumour Materials in Liquid Nitrogen*

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SOME YEARS ago, we reported our results with the preservation of tumour material at −70°C using dry ice [1]. These results were not uniform in that −70°C was insufficient to preserve all the tested tumour strains over a long period of time. It was especially irritating that the material survived the freezing process but was not transplantable after some weeks of storage. During recent years liquid nitrogen has been used more and more for the preservation of different materials and some months ago Kline *et al.* [2] and Wodinsky *et al.* [3] published their experiences with tumour material.

Based on our results mentioned above we started in 1964 a programme to study the usefulness of equipment supplied by Union Carbide Ltd. for freezing material automatically at slow rates of temperature decrease and employing liquid nitrogen to maintain the deep frozen material. Since our tumour collection contains more than forty strains in different kinds of animals, their preservation is very important especially from an economical point of view.

MATERIAL AND METHOD

We preferred the transplantation of tumour-brei minced by an "Embryonenquetsche" (Fa. Bergmann KG., Berlin—Bad Soden/Ts, Germany). The minced material was suspended in normal Hanks balanced salt solution 1:1 without addition of serum. The preservants were glycerol 5% or Dimethylsulphoxide DMSO) 7·5%. This mixture was placed in plastic tubes (Falcon Ltd) in 4-ml quantities and sealed by a sealing apparatus. After filling and sealing the material was stored in an ice box at +4°C for 30 min before being introduced into the BF-3 freezing apparatus. The whole equipment for the deep-freezing procedure consists of 1. thermal sealer (model CT-1); 2. liquid nitrogen container (LS 160 B); 3. freezer (BF-3-1); 4. freezer controller (BF-3-1); 5. electronic controller (electr. 18); 6. liquid nitrogen refrigerator (model LR 120). Prior to use the samples are thawed rapidly in a 37°C water bath.

RESULTS

In the first experiments the material was frozen automatically at a rate of 1°C/min as described in the information supplied by the Union Carbide Ltd. It is mentioned by the manufacturers that a super-cooling effect can happen occasionally. On the contrary, in our hands this phenomenon occurred regularly as shown in Fig. 1. The supercooling effect is characterized by an interruption of the temperature fall with subsequent rise for some minutes. Thereafter a sudden fall of temperature is observed of up to 5°C during a few seconds. In

*Presented in part at the International Symposium of Refrigeration, Prague, September 9 (1965).

assays 7 and 8 of Fig. 1 we have the following transplantation results:

	Tumourstrains	Takes
Assay 7	Amelanotic melanoma 4/hamster	0/3
	Benzpyren sarcoma/hamster	1/3
	Renal carcinoma 1/hamster	0/3
	Sarcoma R.F. 63/rat	0/3
Assay 8	Rous sarcoma/rat	0/3
	Ascites hepatoma 602 Yoshida/rat	0/3
	Myelo-sarcoma T 58 (oberling-Guerin)/rat	3/3

It shows, that this event is sufficient to destroy the transplantability of the material. The problem can be solved only by carefully regulating the influx of liquid nitrogen into the deep-freezer by hand under careful observation.

Figure 2 demonstrates two assays using this technique. In assays 45 and 48 we have the following transplantation results below.

After having overcome these difficulties most of our tumour strains could be preserved by using Glycerol (5%) as preservants, as demonstrated in Table 1.

The first part of Table 1 demonstrates the behaviour of rat tumours. Most of them were preserved using 5% Glycerol with the exception of the Yoshida-sarcomas, Sarcoma 2677, Sarcoma 2844 K and the two Shay leukaemias. In the case of these tumours 7·5% DMSO was needed. As seen even 7·5% DMSO was not very effective. This fact was unexpected since the sarcomas have a normal transplantation rate of 95 per cent when transplanted from animal to animal. The Shay leukaemias are not running regularly and therefore difficulties were expected.

The second part of Table 1 summarizes the results obtained with tumours in mice. The results are similar to those of the rat tumours, most of tumours needing 5% Glycerol only for preservation with the exception of the leukaemia SOV 16 (Graffi) and the Lymphosarcoma 4946. Neither are preserved by Glycerol and using 7·5% DMSO the results are not uniform.

In the SOV 16 tumour for example, the same material of origin produces 1/5 takes 10 days after freezing, 3 months later 0/5 and 6 months later 5/5 takes. A second sample tested 10 days after freezing gave 5/5 takes but 1/5 takes 3 months after freezing. With the Leukaemia 4946 we have had similar experiences.

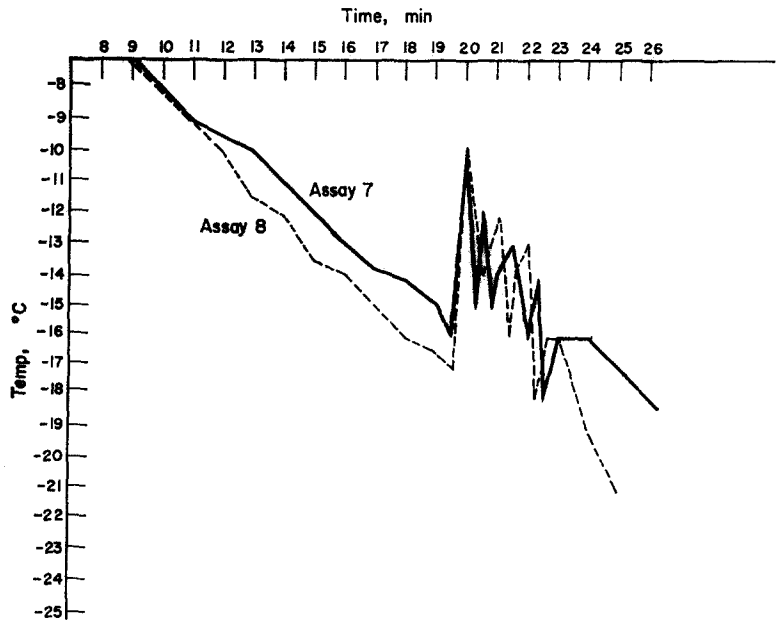

Fig. 1. *Super cooling effect positive.*

	Tumourstrains	Takes
Assay 45	Shay lymphatic leukoma	
	Leukoma/rat	3/5
	Hepatoma 1/hamster	3/5
	Benzpyren sarcoma/hamster	3/3
Assay 48	Leukaemia SOV 16/mouse	3/4
	Lymphosarcoma L4946/mouse	3/5
	Uterus epithelioma Guerin/rat	3/3
	Uterus epithelioma Guerin/rat	3/3

The third part of Table 1 demonstrates the results obtained with tumours in hamsters, rabbits and guinea pigs. These tumours have been frozen with DMSO (7·5%) and all are still viable.

Because the irregular behaviour of the tumours tested we have studied the effect of

Table 1. *Viability of tumour strains after preservation at* −196°C

	Tumour strains	Takes* after preservation of				
		10 days	3 months	6 months	9 months	12 months
I.	Benzpyren Sarcoma Hoechst	3/3	3/3		3/3	3/3
	MurphySturm Lymphosarcoma	5/5	4/4	2/2	3/3	3/3
	Myelosarcoma T 58 (Oberling-Guerin)	3/3	3/3		3/3	3/3
	Rous Sarcoma Hoechst	3/3	3/3	5/5	3/3	4/4
	Shay lymphatic leukoma	2/6		1/7	4/8	
	Shay myeloid leukaemia	7/8		5/10		
	Shay myeloid leukoma	2/4		2/4		
	Uterus Epithelioma Guerin	1/3	3/3		3/3	3/3
	Walker Carcino-Sarcoma I	2/3	3/3		3/3	3/3
	Walker Carcino-Sarcoma III	3/3	3/3		3/3	3/3
	Walker Carcino-Sarcoma BASF (Thio-TEPA resistant)	3/3	4/4			
	Yoshida Sarcoma	3/3	3/3		3/3	
	Yoshida Sarcoma (Tetramin-resistant)	3/3	3/3		4/4	
	Ascites-Hepatoma 130 Yoshida	2/3	3/3		3/3	3/3
	Ascites-Hepatoma 602 Yoshida	2/3	3/3		2/3	3/3
	Sarcoma 2677 (Methylchol. transplant. Hoechst)	3/3	1/3		2/3	2/3
	Sarcoma 2844/K (Aminofluoren transplant. Hoechst)	3/3	1/3		2/3	3/3
	Jensen Sarcoma	3/3		4/4		
	Gold Sarcoma BASF	3/3		4/4		
	Novikoff Hepatoma (ascitic form)	3/3	4/4			
	Novikoff Hepatoma (solid form)	3/3				
	Sarcoma RF 63	2/3	2/3		3/3	3/3
	Adenocarcinoma 29 316/64 (DMBA, transplant. Hoechst)	3/3				
II.	Ehrlich Ca, solid	3/3	3/3		3/3	3/3
	Ehrlich Ca, ascitic	5/6	5/5	6/6	6/6	4/6
	Krebs 2-Ca	5/5	5/5	5/5	5/5	7/7
	Sarcoma Sa II Landschütz	5/5	5/5			
	Sarcoma 180	5/5	5/5	5/5		7/7
	Rous Sarcoma	4/5	5/5	5/5		6/7
	Harding-Passey Melanoma	4/5	5/6	5/6		6/7
	Harding-Passey Melanoma BASF	1/6	3/5			
	Leukaemia L 1210	4/4		2/2		
	Friend-Virus Leukaemia	4/5	6/6	5/8		5/8
	Leukaemia SOV 16	1/5	0/5	5/5		1/5
		5/5	1/5			
	Lymphosarcoma L 4946	1/5			3/5	
		5/5		0/2		
				2/5		
		3/5	0/5			
	Nemeth-Kellner Lymphoma	5/5	4/4	5/5		
	PCG Plasmacytoma	4/4	5/5			
III.	Amelanotic Melanoma 3	3/3			3/3	4/4
	Adrenal Carcinoma 4	3/3			2/3	5/5
	Amelanotic Melanoma 4	3/3			3/3	4/4
	Hepatoma 1	3/3			3/3	5/5
	Renal Carcinoma 1	3/3		3/3		
	Benzpyren Sarcoma	3/3			3/3	3/3
	Myxosarcoma (spontaneous) (Hoechst)	3/3			2/3	2/3
	Brown-Pearce Carcinoma	2/2	2/2	3/3		2/3
	VX-2 Carcinoma	2/2	2/2	3/3		3/4
	Daels-Sarcoma	3/3	4/4	2/3		

*The numerator expresses the number of takes, the denominator expresses the number of animals used.

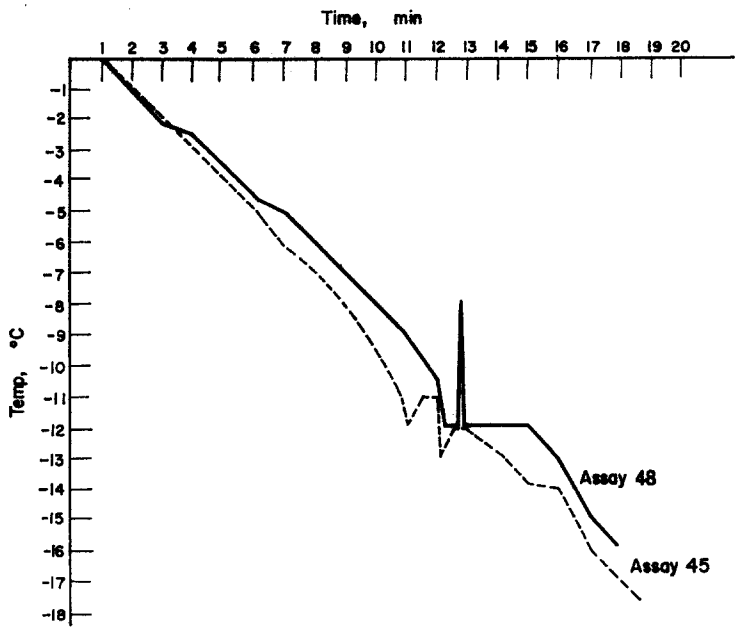

Fig. 2. Super cooling effect negative.

higher glycerol and DMSO concentrations, but 10–20% glycerol and 10–40% DMSO gave no better results. As mentioned above we diluted the tumourbrei with Hanks balanced salt solution 1: 1, therefore the salt concentration in the final mixture is 50% only. We have tried a Hanks medium of double the concentration, but the results were the same.

Studies are now in progress using other preservatives such as propylenglycol, diethylencarbonate and hemaccel® (Behringwerke AG., Marburg, Lahn). Finally we are studying the possibility of fast freezing and it seems that ascitic tumours can be preserved by this method.

DISCUSSION

Our results demonstrate that the simple composition of the freezing medium is sufficient to preserve the most of our tumour strains. Nevertheless it seems that it is insufficient to overcome the difficulties of the supercooling effect observed during automatically directed deep-freezing procedures using the BF-3 freezer. Both Wodinsky and Kline [2, 3] used the same freezing medium, which was different from ours, consisting of Eagle's medium, Hank's balanced salt solution and horse serum. Those authors did not report on the deleterious effect of supercooling on the transplantability of their tumour material.

Another difference between our method and that of Wodinsky is in the quantity of the material used, 1·5 ml in Wodinsky's [3] and 4 ml in our assays. These factors may influence the results. This conclusion can be drawn from our studies with the rat sarcomas. In case of the leukaemias in mice and rats it may be that these tumours are more sensitive to the freezing process. Therefore, Kline [2] has proposed the use of spleen cells as preserving additive.

Acknowledgement — We wish to thank Mrs. Elfriede Brand and Mrs. Ingeborg Schlehuber for their technical assistance.

RÉSUMÉ

Grâce à l'étude de plus de 40 souches de tumeurs apparues dans différentes espèces animales, on a constaté qu'un appareillage consistant en un congélateur BF-3 avec azote liquide et additifs, facilite la congélation lente du matériel. Lors de la congélation automatique, on a toutefois constaté un effet de surfusion nuisible à la viabilité du matériel. On peut cependant pallier cet inconvénient en réglant, à la main, l'entrée de l'azote liquide dans le congélateur, pendant la période de température critique. Nous utilisons un moyen de congélation très simple: il se compose de la solution salée et équilibrée de Hanks, à laquelle nous ajoutons soit 5% de glycérol, soit 7,5% de DMSO. Nous pouvons ainsi conserver la plupart, mais non toutes les souches de tumeurs. C'est pourquoi nous poursuivons nos recherches en vue de résoudre ce problème.

SUMMARY

Testing more than forty tumour strains from different kinds of animals and of different types, we found that equipment consisting of a BF-3 freezer with additives and liquid nitrogen is useful for freezing down the tumour material at low rates. A supercooling effect has been observed which is deleterious for tumour viability when freezing is carried out automatically. It can be overcome by regulating the influx of liquid nitrogen into the deep freezer by hand during the critical times. The simple freezing medium used consisting of Hanks balanced salt solution with the addition of Glycerol (5%) or DMSO (7·5%) only is sufficient for most of the tumour strains tested, some of which however cannot be preserved with this preservation process. Studies are in progress to solve this problem.

ZUSAMMENFASSUNG

Wir haben über 40 verschiedene Tumorstämme verschiedener Tierarten bei—196°C zu konservieren versucht und haben gefunden, daß zum Einfrieren des Materials und zur Aufbewahrung bei—196°C eine Apparatur, die aus dem BF-3 Einfriergerät und Zusätzen besteht, gut brauchbar ist. Das Tumormaterial wurde unter Verwendung von flüssigem Stickstoff langsam eingefroren.

Es wurde ein "supercooling effect" beobachtet, der beim automatischen Einfrieren auftritt und der die Lebensfähigkeit des Tumormaterials zerstört. Dieser "supercooling effect" kann ausgeschaltet werden, wenn man in der kritischen Temperaturzone den Zufluß von flüssigem Stickstoff in das Einfriergerät mit der Hand regelt.

Das von uns benutzte einfache Einfriermedium besteht aus Hanks-Lösung und hat nur einen Zusatz von 5% Glycerin oder von 7,5% DMSO. Damit konnten die meisten Stämme konserviert werden.

Bei einigen Stämmen ist das Verfahren ungenügend, und Untersuchungen zur Lösung dieses Problems sind im Gange.

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Announcement

Letters to the Editor

Beginning with No. 3, the European Journal of Cancer will accept "letters to the Editor" for prompt publication. The length of these short communications should not exceed 750 words including title, authors, bibliography and acknowledgements. They should furnish sufficient data to support their conclusions and conform to the usual standards of the Journal.

Lettres à l'Editeur

A partir du n° 3, le Journal Européen de Cancérologie acceptera, en vue d'une publication rapide, de courtes notes appelées "lettres à l'Editeur". Leur longueur ne doit pas dépasser 750 mots, y compris le titre, les noms d'auteurs, la bibliographie et les autres mentions. Elles devront fournir les données expérimentales ou d'observation nécessaires pour étayer leurs conclusions et se conformer aux instructions applicables aux articles ordinaires.

Briefe an den Herausgeber

Beginned mit der 3. Folge wird die "Europäische Zeitschrift für Cancerologie" "Briefe an den Herausgeber" für baldige Veröffentlichung annehmen.

Der Umfang dieser kurzen Mitteilungen sollte 750 Worte nicht überschreiten, einschliesslich Ueberschrift, Autoren, Bibliographie und andere Annerkungen. Sie sollten genügend Unterlagen enthalten, um die Schlussfolgerungen zu untermauern und allen übrigen Anforderungen der Zeitschrift entsprechen.

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Protection Against the Toxicity of Alkylating Agents by Thiols: The Mechanism of Protection and its Relevance to Cancer Chemotherapy

A REVIEW

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FOLLOWING the discovery that chemicals could protect whole animals against radiation induced toxicity [1, 2], it was soon demonstrated that thiols could protect rats and mice against the toxicity of the alkylating agent HN2 (methyl di-2-chloroethylamine) [3]. Protection against alkylating agent toxicity was a logical extension of the work on radiation protection since it was believed that alkylating agents were closely related to X-rays in their mechanism of cytotoxicity and hence their terminology "radiomimetic agents" [4].

Thiol protection against radiation and alkylating agents

While there is a superficial resemblance between the protection thiols give to animals against the toxicity induced by either irradiation or alkylating agents, there are also significant differences. Table 1 shows the effectiveness of three thiols in protecting rats or mice against X-rays or against the alkylating agents HN2 and merophan (*o*-di-2-chloroethylamino-DL-phenyl alanine). The expression of the protective effect in terms of a dose reduction factor enables a quantitative comparison to be made between these thiols, which in all experiments summarized in Table 1, have been employed at their maximum tolerated doses.

Table 1. *Comparative protection given by three thiols against X-rays or alkylating agents*
(Dose reduction factors)*

	X-rays	HN2	Merophan
Cysteamine (M.T.D, 150 mg/kg)	[6] 1·8–2·0	[16] 2·0	[8] 1·3
AET (M.T.D, 200 mg/kg)	[5] 1·8–2·0	[15] 2·0	[8] 2·0
Cysteine (M.T.D, 1000 mg/kg)	[7] 1·8	[3] 7·0	[8] 4·2

*There is a variation in the dose reduction factors found for different strains of animal. These figures are the highest dose reduction factors recorded in the literature. The dose reduction factor (DRF) is obtained from the ratio; LD_{50} (or LD_{99}) of animals pretreated with the thiol/LD_{50} (or LD_{99}) of animals irradiated or given the alkylating agent only. The thiol is given at the previously determined optimum time to give the greatest protection, usually 30–5 min before the alkylating agent or irradiation. In most cases thiol pretreatment does not alter the slope of the dose–mortality curve so that the DRF obtained applies to all doses of X-rays or alkylating agents.

It appears that in radiation protection experiments the maximum dose reduction is about 2. AET [5], and cysteamine [6] are probably

slightly better protectors than cysteine [7] although their maximum tolerated doses are lower. Against HN2 however it is quite clear that under optimal conditions cysteine has the best protective effect, dose reductions of 7 or greater having been obtained for rats and up to 4 for mice [3]. This difference in protective ability between cysteamine and cysteine is further demonstrated in experiments using the aromatic nitrogen mustard merophan [8]. Cysteamine is definitely non-protective to rats treated with this agent while cysteine gives a dose reduction of four. These differences between cysteine and cysteamine certainly cannot be accounted for by variation in strain of animal used and other experimental variables since Goldenthal *et al.* [9] have made a careful comparison of the effectiveness of cysteine and cysteamine in protecting rats both against radiation and HN2. In the rats they used, cysteamine gave good protection against irradiation but little protection against HN2. Cysteine on the other hand protected the rats both against radiation and HN2.

From these results it is not reasonable to assume that those mechanisms by which chemical protection against radiation is mediated, are necessarily those which apply for chemical protection against the alkylating agents.

Mechanism of action of the alkylating agents

The pharmacological properties of the thiols recorded in the literature are many and varied. Any one of these many effects may play a role in protecting against the toxicity of the alkylating agents. It is fortunate however, that owing to their importance in cancer chemotherapy, a great deal is now known about the mechanism of action of the alkylating agents [10]. Formula 1 shows a typical alkylation reaction, which may be considered to be the replacement of the hydrogen atom of a molecule by an alkyl ($R\mathrm{CH_2}-$) group:

$$R.\mathrm{CH_2}\,X+\mathrm{H}R'\longrightarrow R.\mathrm{CH_2}R'+\mathrm{H}X \qquad (1)$$

The radical R may be a compound one, the only requirement being that the carbon atom through which the attachment to the molecule R' is made is a fully saturated one. Also under alkylation may be included reaction of the agent with a tertiary amine to form a quaternary ammonium compound (formula 2), or reaction with an ionized acid to form an ester (formula 3).

$$R.\mathrm{CH_2}.X+NR'_3\longrightarrow R.\mathrm{CH_2}.\overset{+}{N}R'_3+X^- \qquad (2)$$

$$R.\mathrm{CH_2}X+\bar{\mathrm{O}}\mathrm{OC}R'\longrightarrow R.\mathrm{CH_2}.\mathrm{OOC}R' \qquad (3)$$

$$R_2\mathrm{N}.\mathrm{CH_2CH_2Cl}\longrightarrow R_2\mathrm{N}.\mathrm{CH_2CH_2}^{+}\xrightarrow{'R^-} R_2.\mathrm{N}.\mathrm{CH_2CH_2}R' \qquad (4)$$

The alkylation reaction of one type of alkylating agent is shown in formula 4. The basic nitrogen atom causes dissociation of the chlorine atom with the effective formation of a transient, highly reactive, carbonium ion (R_2.N .$\mathrm{CH_2CH_2}^+$). This reactive intermediate will combine with molecules containing negatively charged atoms or atoms of high electron density. These sites, very reactive towards alkylating agents are termed nucleophilic centres. In the animal there are large numbers of such nucleophilic centres; the ionized thiol and carboxyl groups and uncharged amino groups of enzymes and structural proteins; the ionized phosphate groups and ring nitrogens of nucleotides and nucleic acids. Free amino acids and peptides, vitamins, co-factors, steroids and lipids all possess nucleophilic centres capable of reacting with alkylating agents. There is now good evidence however, that the alkylating agents which have tumour inhibitory activity owe their characteristic cytotoxicity to a direct reaction with DNA [11].

Figure 1 illustrates events taking place when an alkylating agent is administered to an animal. A large number of sites will be in a form available for reaction with the agent, and it is well known that even at physiological dose levels, numerous reactions take place with different molecules. Yet it is thought that despite these various alkylations, cytotoxicity

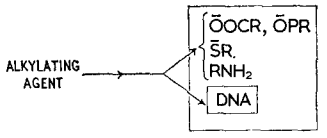

Fig. 1. *Some cell sites sensitive to alkylating agents.*

will be manifest only when the appropriate level of DNA alkylation takes place. It is important to recognize that at physiological dose levels, the amount of alkylating agent administered is extremely small when compared with the large number of molecules available for alkylation, so that in fact only a small fraction of available sites will be alkylated. Two important factors will govern which sites are alkylated:

(a) The concentration of the particular site. The greater the concentration the more will be alkylated, and
(b) The affinity of the site for alkylation by the agent.

Some compounds (e.g. ionized thiols) are extremely reactive towards alkylating agents while other molecules (e.g. amines) also react with alkylating agents, but at a much slower rate. Both types of compound in the presence of excess alkylating agent will undergo complete reaction. On the other hand where the two nucleophilic compounds are in excess (as would occur *in vivo*), a greater proportion of the more reactive compound will be alkylated. The affinity of a particular molecule for reacting with alkylating agents may be measured chemically and expressed as the competition factor. At equal concentrations of their reactive forms, compounds with high competition factors will react to a greater extent with an alkylating agent, than compounds with low competition factors, when there is only a limited amount of alkylating agent available. The protective thiols all have high competition factors for alkylating agents. If these thiols after injection, remain in the animal in an available form (i.e. they are not bound to protein, oxidized or excreted), then it seems evident that they will react to a certain extent with an administered alkylating agent. The added thiol will in fact compete with cell sites for alkylation by the small amount of agent entering the animal. Reaction of the alkylating agent with the exogenous thiol will reduce the amount of agent available for reaction with DNA and other cell sites. A decreased amount of DNA alkylation will lead to a reduction in cytotoxicity and the thiol will be acting as a protective agent. I would like to consider whether this direct interaction between the thiol and the alkylating agent (which may be termed "competitive removal") can account for all the protection observed in experiments reported in the literature.

Properties of protecting agents

If competitive removal is the major mechanism by which chemical protection against the alkylating agents is achieved we may predict, from the consideration above, two main properties of protecting chemicals. (a) They should contain nucleophilic centres with a high competition factor for alkylating agents. (b) They should attain a high concentration in the animal in an available form, at protective dose levels. To achieve a high concentration the compound must be relatively non-toxic enabling the administration of large doses. It is of interest to see how the known chemical protectors fulfil these requirements. Ogston [39] has measured the competition factors of a number of compounds for sulphur mustard under approximate physiological conditions. Five of these compounds have also been used in attempts to protect animals against the toxicity of HN2 and it is expected that their competition factors for HN2 will be similar. In Table 2 an attempt is made to correlate the protection a thiol gives to an animal, with the competition factor of the thiol and the dose of the thiol required to give the protection. Such an attempted correlation is made difficult because of the variation in protection obtained for any particular thiol. This variation is probably due to the animals used, to the route and time of administration of the thiol and to the amount given. However if only the maximum dose reduction factor recorded in the literature for any one thiol is taken (the assumption being made that the optimal conditions for protection were used in this experiment) a good correlation is seen. Table 2 shows the maximum dose reduction factors recorded for each thiol in protecting animals against the toxicity of HN2, and the dose of the thiol required to give this protection. In all cases the dose of the thiol has been at the maximum tolerated level. Also shown is the competition factor of the thiol. It can be seen that cysteine [3, 12] and thiosulphate [12, 13] have given the best protection and this agrees well with their properties of low toxicity and high competition factor. Thiourea [14] however, while no more toxic than cysteine or thiosulphate is only a moderate protector, and this might have been predicted since its competition factor is lower. Conversely sodium diethyl dithiocarbamate [17] while having a high competition factor is more toxic than cysteine and is predictably only a moderate protector. Dimercaptopropanol (BAL) has

Table 2. Chemicals used in attempts to protect rats or mice against the toxicity of HN2

Chemical	M.T.D (mg/kg)	Competition factor	D.R.F.
L-cysteine	1000	$1{\cdot}2\times10^{3}$	7·0
Thiosulphate	2000	$2{\cdot}7\times10^{4}$	5·0
Thiourea	1200	$1{\cdot}5\times10^{2}$	2·0
Diethyldithiocarbamate	350	$3{\cdot}4\times10^{4}$	2·0
Dimercaptopropanol	100	$7{\cdot}8\times10^{2}$	1·0

been used many times in protection experiments with little success [3, 8, 18, 19], and this may be attributed to a combination of high toxicity and fairly low competition factor. It is also of interest that every compound listed in Table 2 is water soluble, and the best results have been seen when the compounds have been given subcutaneously or intraperitoneally. These conditions (i.p. in water) are usually more effective in obtaining high tissue concentrations of chemicals than administration by mouth or in oily solvents or suspensions.

The availability of injected cysteine

The low toxicity of a compound is only an indication that it may, by giving high doses, be possible to achieve a high body concentration. One can imagine situations where even after administration of high dose levels a chemical may be so quickly dealt with by metabolism or excretion that it never reaches a significant concentration. For interaction between the protecting chemical and alkylating agent to take place to any significant extent a high concentration of the protecting chemical in an available form is necessary. Measurements have been made of the level of cysteine in the tissues of the rat following intraperitoneal administration [20]. This work was initiated by the finding that cysteine protected against the toxicity of merophan only if doses of 250 mg/kg or greater were given. Yet above this dose level the extent of protection was directly related to the log dose (Table 3). The rise in the protein-free SH level of spleen following the administration of different doses of cysteine is shown in Fig. 2. The protein-free SH level (i.e. the SH level in the trichloracetic acid soluble fraction of tissue homogenates) almost certainly represents thiols available for reaction with alkylating agents. The SH levels shown in Fig. 2 were obtained by an amperometric method and represent the total SH content of the soluble fraction. These values could be misleading since they probably include thiols (such as glutathione) with low competition factors, and therefore not very reactive towards alkylating agents. Recently however Ball [21] has shown that the rise in free SH occurring in tissues after administration of cysteine is probably due entirely to the accumulation of cysteine itself. The curves shown in Fig. 2 may hence be assumed to represent free cysteine levels, where

Table 3. The protection given by various dose levels of cysteine to rats treated with merophan

Dose level cysteine (mg/kg)	LD_{50} merophan	D.R.F.
None	3·65	—
62·5	3·99	1·0
125	3·99	1·0
250	6·50	1·8
500	11·02	3·0
1000	15·24	4·2

In this experiment female Wistar rats were used. They were given the cysteine i.p. in aqueous solution 30 min before the alkylating agent which was also given i.p. in aqueous solution.

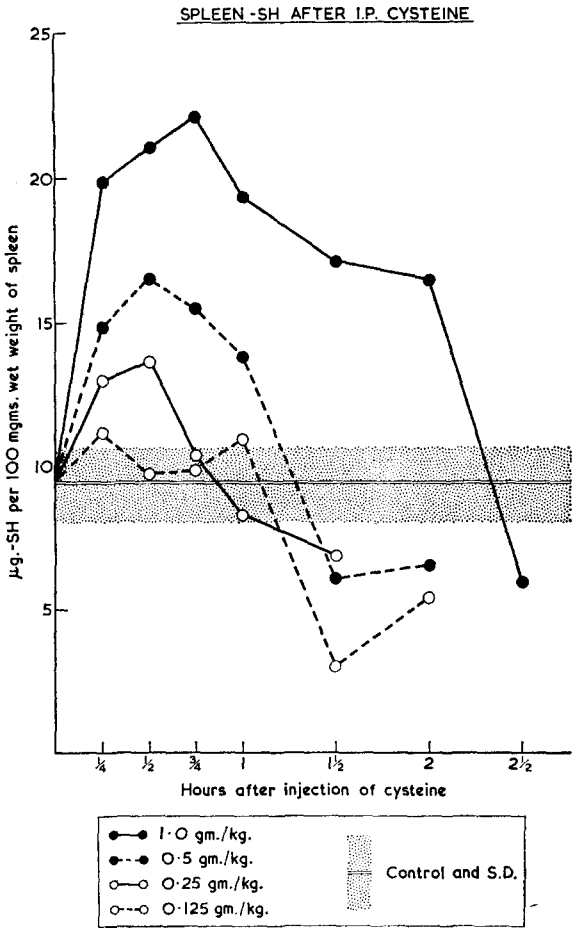

Fig. 2. The rise in protein free SH level of rat spleen following intraperitoneal administration of different doses of L-*cysteine. Protein free SH was estimated in the trichloroacetic acid soluble fractions of spleen homogenates by an amperometric method using excess* p-*chloromercuribenzoate and back titrating with cysteine* [20]. *If the protein free SH level is determined using Ellman's reagent, identical results are obtained. Using the glyoxylic acid method which distinguishes cysteine from glutathione, the rise in protein free SH occurring after injection of cysteine has been shown to be due entirely to the accumulation of cysteine itself* [21].

the level rises above normal. Most tissues show patterns similar to that seen in spleen. The level is high soon after injection (15 min was the first point taken in the experiment illustrated, but the level is also high at 5 min [21]), and remains high for 45 min before it begins to fall to normal. There is a tendency for the maximum concentration to occur at 30 min, but while this is characteristic for bone marrow it is not always seen in other tissues. The different levels of free cysteine accumulating in spleen after administration of various doses fits in well with the explanation of protection by competitive removal. The highest dose of cysteine leads to the greatest accumulation of cysteine in tissues and gives the best protection. Two lower doses of cysteine (500 and 250 mg/kg) give successively lower concentrations and less protection; 125 mg/kg of cysteine and lower doses lead to no concentration of the free thiol in tissues and no protection is seen. Also after 1 g/kg of cysteine, protection is good if the alkylating agent is given no later than 45 min after thiol pretreatment, i.e. when the tissue cysteine concentration is high. If administration of the alkylating agent is delayed to 45–90 min after pretreatment the protection is progressively less. Little protection is observed when the alkylating agent is given later than 2 hr after the thiol, i.e. at a time when the injected cysteine has been removed from tissues (Table 4).

Table 4. *Effect of altering the pretreatment time on the ability of cysteine to protect rats against the toxicity of merophan*

Time of cysteine pretreatment (min)	D.R.F.
15	>4·2*
30	4·2
60	3·7
120	1·6

Female Wistar rats. 1 g/kg cysteine was given i.p. in neutral aqueous solution at various times before merophan which was also given i.p. in aqueous solution.

*Some of the injected cysteine is still present in the peritoneal cavity at the time the merophan is given.

Reactivity of alkylating agent and degree of protection

Different alkylating agents react with a particular nucleophilic centre at different rates. For many agents their rate of reaction with water has been measured and expressed as the "half-life" of the agent. Since after injection, cysteine remains in the animal for a relatively short period, it would seem that there would be more interaction between the thiol and a quick acting agent than between the thiol and a slower acting agent. The rate of reaction of most alkylating agents can be greatly influenced by a number of factors and the measurement of chemical half-life can only be taken as an approximate estimation of their expected rate of reaction *in vivo*. Nevertheless it is of interest to see that cysteine has been particularly successful in protecting against HN2 and merophan which have short half lives (i.e. they react quickly), less effective against melphalan [22] with a longer half life and inactive against a range of alkylating agents with half lives in excess of 2 hr (Table 5) [8, 9, 22, 23].

Table 5. *The effect of cysteine in protecting animals against the toxicity of alkylating agents of different half-lives*

Alkylating agent	D.R.F.	Approximate half-life
HN2	7	15–30 min
Merophan	4	
Melphalan	2	1–2 hr
Leucyl melphalan	2	
Benzoic acid mustard	1	
Ethylene-imides	1	>4 hr
Myleran	1	
Eponate	1	

The half-life of these agents may be measured by a number of methods . Usually the rate of formation of hydrogen ions under standard condition is measured (e.g. pH 7·5, 37°C). This rate may be influenced by a number of factors. The chloride concentration of serum would reduce the reactivity of the 2-chloroethylamines (nitrogen mustards) while the rate of reaction of many of the agents would be influenced by the concentration of nucleophilic centres.

Studies with labelled alkylating agents

Direct evidence for an interaction between administered thiols and alkylating agents *in vivo* should be obtained from studies using labelled materials. Refined methods for the isolation of pure nucleic acid and protein fractions of cells have been published and it might be a relatively simple experiment to show that in chemically protected animals there was less binding to these fractions and an excretion of the alkylated protecting thiol. Nevertheless there are only a few reports in the literature where labelled alkylating agents have been used to study the mechanism of protection. Contractor [16] and Goldenthal *et al.* [9] have both measured the

excretion in the urine of C^{14} HN2. The results are summarized in Table 6. Rats given C^{14} labelled HN2 excreted (in the urine) 15 per cent of the radioactivity administered, in 24 hr. The same rats pretreated with cysteine and well protected excreted 43 per cent of the total label

Table 6. *Percentage of total radioactivity recovered in the urine after administration of C^{14} labelled HN2 to animals with or without thiol pretreatment*

	Contractor (%)	Goldenthal *et al.* (%)
HN2 alone	64	15
HN2 after thiol pretreatment	95	43

administered in 24 hr. Contractor, using mice protected with cysteamine (dose reduction 2) showed that there was 64 per cent excretion of label in mice given HN2 alone while in the cysteamine pretreated animals the total radioactivity in the urine in 24 hr was 95 per cent. These two results show essentially the same phenomenon, yet the two groups interpret the results in a different manner. Contractor believing cysteamine to protect by mixed disulphide formation assumes that in the presence of the protector, HN2 can no longer alkylate the protected sites. It therefore undergoes hydrolysis and not being bound to any cell site is excreted more rapidly. This implies that cysteamine is shielding all the many different types of centres reactive to HN2 which are normally present in a vast excess in the animal. Goldenthal *et al.* interpret their results quite differently. They believe that the increased rate of excretion of label in the protected animals is caused by the HN2 reacting preferentially with free cysteine rather than binding to other cell sites. In support of this view they show that the increased amount of labelled material excreted in protected animals is a reaction product of cysteine with HN2. More recently we have obtained direct evidence that cysteine can prevent the nitrogen mustard melphalan (*p*-di-2-chloroethylamino-L-phenyl-alanine) from reacting with cell sites [37]. Melphalan at a dose of 0·21 mg/kg causes 90 per cent inhibition of the solid Yoshida sarcoma growing in Wistar rats. In rats bearing the same tumour but pretreated with cysteine (1 g/kg, i.p., 30 min beforehand) a dose of 0·46 mg/kg of melphalan is required to cause 90 per cent tumour inhibition. The ratio of the two doses is about 2 and is the dose reduction factor for the tumour. If rats with the Yoshida tumour are given melphalan labelled with tritium, various cell fractions can be isolated and a measurement of the radioactivity of each fraction indicates the degree of reaction with melphalan. The reaction of melphalan with the nucleic acids and proteins of the Yoshida tumour following administration of labelled melphalan is shown in Table 7. If animals are pretreated with cysteine (1 g/kg, i.p. 30 min beforehand) and the same dose of labelled melphalan given it can be seen that the reaction with nucleic acids and nucleoproteins is much

Table 7. *Effect of cysteine on the reaction of melphalan with various cell fractions of the Yoshida tumour in rats*

Fraction	Count/min per mg	
	Control melphalan only	Pretreated 1 g/kg cysteine
DNA	139	23
RNA	174	40
Nuclear protein	165	62
Cytoplasmic protein	283	281

H^3-Melphalan 30 mc/mmole was given 4 mg/kg i.p. In the pretreated groups the animals received cysteine 1 g/kg, i.p. in neutral aqueous solution 30 min before the melphalan. Cell fractions were isolated according to the method of Kirby [40], 1 hr after the injection of melphalan. After dissolving in perchloric acid (DNA,RNA) or sodium hydroxide (protein) radioactivity was measured in a liquid scintillation counter.

reduced. In fact although this dose of cysteine protects the tumour two-fold there is a five-fold reduction in the reaction with nucleic acids and nucleoprotein. In the case of cytoplasmic protein cysteine has had little effect, but the radioactivity associated with this fraction represents mainly adsorbed rather than alkylated material. Since the amount of label associated with this fraction is relatively unaltered by cysteine pretreatment it shows that the thiol is having very little effect on the uptake of the alkylating agent by the cell. In model experiments we have also shown that, using physiological conditions, cysteine can reduce the reaction of melphalan with DNA and RNA in solution. Under these conditions the only possible mechanism of protection is one of competitive removal of the alkylating agent and the degree of protection given to the DNA or RNA by the thiol is of the same order as that seen *in vivo*.

Mechanism of alkylation

Early on in the work on chemical protection against alkylating agents it was established that cysteine and thiosulphate both gave good protection to animals treated with HN2. It was subsequently shown that although cysteine protected against the toxicity of merophan to the same extent, thiosulphate was virtually unprotective (Table 8). This difference between thiosulphate and cysteine may be explained in terms of the competitive removal theory.

Table 8. Comparative protection given by cysteine and thiosulphate against the toxicity of HN2 or merophan

	Dose reduction factors	
	HN2	Merophan
Cysteine	5·2	4·2
Thiosulphate	5·2	1·4

The alkylating agents react by one of two mechanisms and a detailed explanation is given by Ross [10]. It may be stated briefly that the first, or SN1 mechanism, relies upon the formation from the agent of an intermediate carbonium ion which is extremely reactive towards either water or other nucleophilic centres, and will react almost immediately it is formed. Merophan acts primarily by this mechanism:

$$\underset{\text{(alkylating agent)}}{RX} \longrightarrow \underset{\text{(carbonium ion)}}{R^+ + X^-}$$

$$\underset{\text{(nucleophilic centre)}}{A^- + R^+} \longrightarrow \underset{\text{(alkylated product)}}{RA}$$

The alkylating agent as such is not reactive but once it has ionized, the carbonium ion it forms is extremely reactive.

The second mechanism, the SN2 type, is shown by the majority of alkylating agents and HN2* is included in this group. In this class the alkylating agent itself is reactive towards nucleophilic centres and alkylation takes place by the initial formation of a transition complex between the alkylating agent and the nucleophilic centre. Dissociation then takes place to give the alkylated product.

The important distinction between the two types of reaction is that the rate of alkylation of SN2 reactors is markedly influenced by the concentration of nucleophilic centres. The higher the concentration of nucleophilic centres the greater will be the number of transition complexes formed and the alkylating agent will react at a much faster rate. With SN1 reactors however since the rate limiting step is formation of the reactive intermediate (the carbonium ion), an increased concentration of reactive centres will not influence the rate of reaction.

The differences between cysteine and thiosulphate as protectors can be explained by the fact that whereas cysteine is freely diffusible, thiosulphate does not enter cells [24]. After administration, thiosulphate accumulates in the vascular system and extracellular spaces of the animal. In an animal pretreated with thiosulphate and then given HN2, the alkylating agent which passes through the vascular system and the extracellular fluid before entering cells is exposed to a high concentration of a nucleophilic centre (thiosulphate) before it reaches cellular DNA. In the presence of this high concentration of thiosulphate a higher proportion of HN2 reacts extracellularly, less unreacted HN2 will therefore enter cells and there will be a reduction in DNA alkylation which will result in a protective effect. Merophan, however, reacts by an SN1 mechanism. If given to an animal pretreated with thiosulphate, the high concentration of thiosulphate in the extracellular spaces can have no influence on the rate of reaction of the alkylating agent. Consequently the same amount of merophan will enter cells as in the animal not pretreated with thiosulphate. Inside the cell there will be no thiosulphate present to compete with DNA for the carbonium ions formed by merophan, and the alkylation of DNA will be the same as in animals not pretreated. No protection will be expected since the level of DNA alkylation will be unaltered.

It should be made quite clear that competitive removal of an alkylating agent can involve two mechanisms. In the first case, illustrated by

$$\underset{\text{(alkylating agent)}}{RX} + \underset{\text{(nucleophilic centre)}}{A^-} \longrightarrow \underset{\text{(transition complex)}}{A^{\delta-} \cdots R \cdots X^{\delta-}}$$

$$A^{\delta-} \cdots R \cdots X^{\delta-} \longrightarrow \underset{\text{(alkylated product)}}{RA} + X^-$$

*HN2 is a rather specialized case and is discussed more fully by Ross [10].

thiosulphate protection against HN2, the alkylating agent can be made to react before it reaches DNA. In the second case, and this applies particularly to agents reacting by an SN1 mechanism, the reactive intermediate still reaches the DNA in the same concentration whether or not the protective chemical is present. The protecting agent in this case will be effective only if it is so distributed, i.e. close to DNA, that it can compete with the DNA for carbonium ions once they are formed. For the latter case one should only consider the concentration of the protective chemical close to the essential site. A compound achieving a high concentration in the extracellular fluid (e.g. thiosulphate) or even in the cytoplasm may not be protective against agents such as merophan, since although it may react readily with carbonium ions it will not be able to react with those ions which happen to be formed close to nuclear DNA. Carbonium ions are so reactive that they will undergo almost instantaneous reaction with nucleophilic centres (including water), in the region where they are formed. Having made the assumption that it is DNA alkylation only that needs to be taken into account when considering the cytotoxicity of alkylating agents at physiological dose levels, then it follows that only those carbonium ions formed in the vicinity of DNA are important. Other carbonium ions formed in the extracellular fluid, in the cytoplasm and even in the nucleus some distance from DNA will be unimportant, since being so reactive they will have alkylated before reaching the DNA.

It follows that for a chemical to be protective against SN1 reactors it must not only have a high competition factor for the alkylating agent but must also concentrate near DNA. This point has been emphasized since if it is at all possible to make comparisons between radiation protection and protection against alkylating agents, they can only be made where the alkylating agent reacts by an SN1 mechanism. In the case of both the carbonium ion and the free radicals formed by irradiation, reaction takes place almost immediately they are formed. Assuming that for X-rays, as for alkylating agents, there are definite sites which must be damaged for toxicity to occur, the protective chemical must be close to these sites whether protection is mediated by radical scavenging, radical transfer or mixed disulphide formation.

Relevance of thiol protection to cancer chemotherapy

The use of alkylating agents clinically is limited since at doses which affect even sensitive tumours there is nearly always a corresponding toxicity to bone marrow. The finding that thiols protected against bone marrow toxicity [3, 18, 19] was of great interest, since it seemed that this might be a way of improving the selectivity of alkylating agents and would enable their administration in sufficiently high doses to kill all tumour cells present while not causing excessive damage to bone marrow. The protection cysteine gives to the bone marrow of rats against the toxicity of merophan is illustrated in Figs. 3 and 4. A sub-lethal dose of 2 mg/kg caused a marked effect on both the

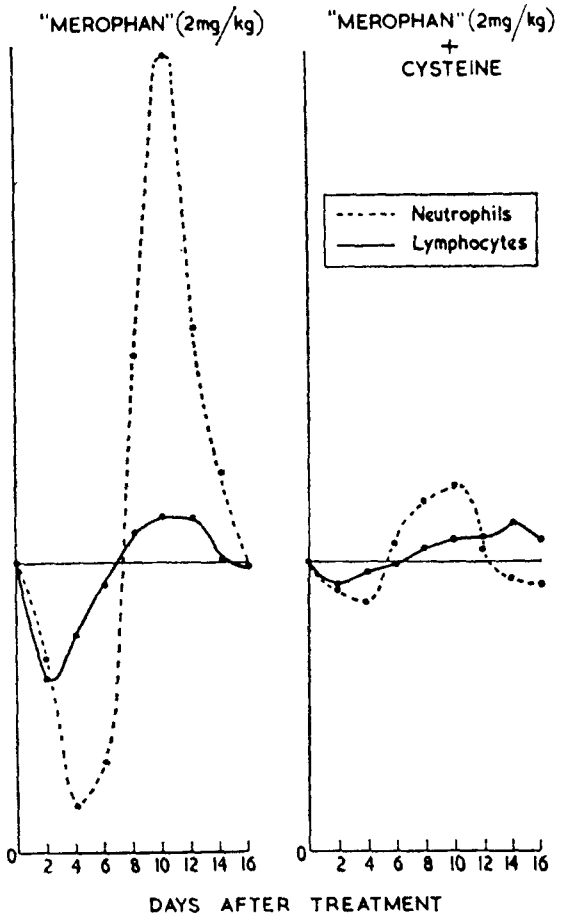

Fig. 3. The effect on blood lymphocytes and neutrophils of the rat following administration of merophan 2 mg/kg. In the animals pretreated with cysteine the thiol was given i.p. 30 min beforehand.

DNA content of bone marrow (Fig. 4) and on circulating peripheral blood cells (Fig. 3). Both these parameters are a measure of bone marrow toxicity [25]. In rats given the same dose of merophan but pretreated with cysteine 1 g/kg these effects are much reduced. In both cases pretreated animals required a dose of 8 mg/kg of merophan before the fall in DNA content of bone marrow or of circulating blood cells was as marked as in animals receiving 2 mg/kg of merophan without pretreatment. This is a dose reduction of four and a similar dose reduction is

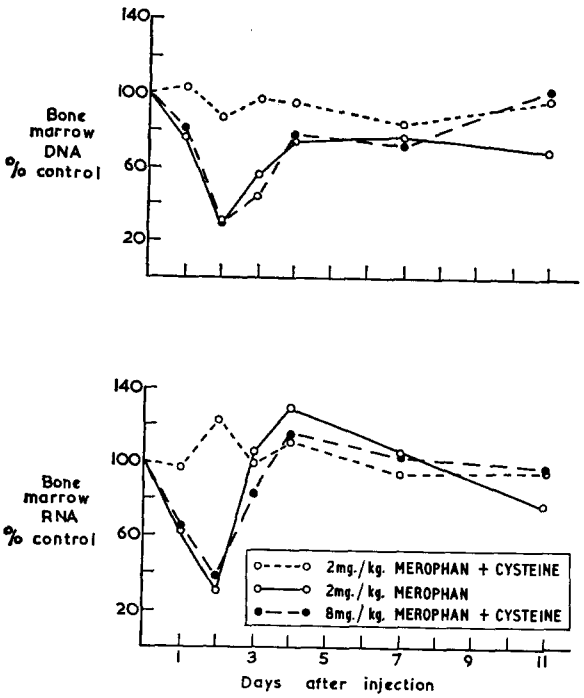

Fig. 4. The effect of merophan on the bone marrow nucleic acid content. Bone marrow was taken from the femurs of Wistar rats and treated essentially by the method of Ogur and Rosen [25] *to obtain a nucleic acid fraction.*
Control values DNA 114 (±19·8) *mg/g dry wt.*
RNA 59·7 (±13·7) *mg/g dry wt.*

obtained in whole body protection experiments. If cysteine pretreatment clinically could improve the selectivity of merophan and other alkylating agents by a factor of four, this would represent a significant advance in chemotherapy. A number of workers have in fact shown that tumour bearing animals can be protected by certain thiols against the toxicity of alkylating agents with no impairment of their anti-tumour action [26–28]. A very large gain in the selectivity of the alkylating agents in these experiments would not be expected, since the thiols used (cysteamine, AET and 1-mercapto-2-aminobutane) are not particularly good protectors of the whole animal. In experiments using merophan, against which animals can be well protected with cysteine, no such gain in the selectivity of anti-tumour action of merophan was apparent [29]. In three tumours studied, while cysteine gave good protection to the host this was paralleled by equal or even greater protection to the tumour (Table 9). As a result there was no increase in selectivity as measured by therapeutic index, and in the case of the Yoshida sarcoma there was a significant loss in selectivity of action of merophan in cysteine pretreated animals. The reason for the failure of cysteine to improve the anti-tumour action of merophan against the Yoshida sarcoma is apparent when the uptake of cysteine by the tumour is compared with the uptake of cysteine by the bone marrow (Fig. 5). The uptake of the thiol by the two tissues with time is similar, but the tumour actually takes up more thiol than the bone marrow. Since

Table 9. Effect of cysteine pretreatment on the therapeutic index of merophan

Tumour	Treatment	ID_{90} (mg/kg)	LD_{50} (mg/kg)	Therapeutic index
ADJ/PC5	Merophan alone	2·3	4·2	1·9
Plasma cell tumour	Merophan + cysteine	8·5	10·6	1·3
Walker	Merophan alone	0·24	3·4	14·2
Carcinoma	Merophan + cysteine	1·6	19·0	11·9
Yoshida	Merophan alone	0·2	3·4	17·0
Sarcoma	Merophan + cysteine	2·2	16·0	7·3

The therapeutic index is the ratio; LD_{50}/ID_{90} (the dose to give 90 per cent tumour inhibition), and is a measure of the selectivity of the drug. The tests were carried out by treating several groups of tumour bearing animals with different dose levels of the drug under test. After a standard time the animals were killed and the tumours dissected out and weighed. The degree of inhibition by any dose level of the drug was obtained by a comparison of the tumour weights of this group with the tumour weights of the control group. If the dose levels are so chosen that the highest dose is lethal and the lowest dose gives little or no tumour inhibition, both the LD_{50} and the ID_{90} (obtained by plotting on semi-log paper) may be determined in one experiment. In the mouse plasma cell tumour, and the rat Yoshida sarcoma experiments, merophan was administered when the tumours were established (10 and 7 days after transplant respectively). For the Walker carcinoma in the rat, merophan was given the day after transplantation. None of these tumours at the time they were treated had any large avascular zones when measured by the lissamine green method. More selectivity might be achieved if older tumours were used and the alkylating agent but not the thiol reached the living cells in the avascular zone.

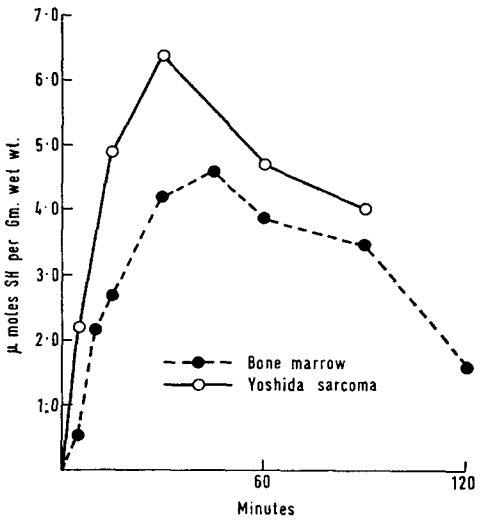

Fig. 5. Increase above normal values of the protein free SH content of rat bone marrow and solid Yoshida sarcoma following the administration of cysteine 1 g/kg i.p. The protein free SH level was estimated in the sulphosalicylic acid soluble fraction of bone marrow and tumour homogenates. The tumours used were taken 7 days after transplantation when their mean weight was 1·5–2·5 g. At 7 days the tumours show no large avascular zones as measured by lissamine green and it is assumed that the thiol concentration throughout the tumour is uniform.
Control values Bone marrow 3·7 μmoles SH/g wet wt.
Yoshida sarcoma 2·1 μmoles SH/g wet wt.

protection is dependent on the intracellular cysteine concentration it must be expected that the tumour will be better protected than the bone marrow (and hence the host) against the toxicity of merophan, and it is not surprising to find that merophan is a less effective anti-tumour agent against the Yoshida sarcoma in cysteine pretreated rats.

If a thiol is to be of use in increasing the selectivity of action of anti-tumour agents, it is evident that it must accumulate in high concentration in normal tissues sensitive to alkylating agents and at the same time not penetrate tumour cells. While this seems just as difficult a problem as finding alkylating agents which selectively accumulate in tumour tissue, it is nevertheless of interest that AET has been shown to concentrate in certain tumours to a lesser extent than in normal tissue [30, 31]. It is significant that AET but not cysteine pretreatment increased the therapeutic index of merophan against the mouse ADJ/PC5 plasma cell tumour [29]. AET has also been claimed to protect tumour bearing mice against the toxicity of HN2 with no reduction of anti-tumour effect [28]. However, since AET gives no higher than a two-fold protection to the host even against a quick acting alkylating agent such as merophan, the maximum increase in selectivity cannot be more than two-fold. Thiols might conceivably be employed systemically to inactivate alkylating agents escaping from the tumour site after close intra-arterial injection or during regional perfusion [32], but again it is not certain whether a high enough dose of thiol could be administered to give a concentration of thiol sufficiently high to be protective.

Sensitivity of tumours to alkylating agents and acquired resistance

An increased intracellular concentration of cysteine or other thiol with a high competition factor for alkylating agents can protect cells against alkylating agent toxicity. If a cell has naturally a high concentration of a thiol or other nucleophile, then one would expect it to be less sensitive to the cytotoxicity of alkylating agents than a cell with a low concentration of the nucleophile yet identical in all other respects. Tumours show a wide variation in their sensitivity towards alkylating agents and while this of course may be due to a combination of many factors, the concentration of nucleophilic centres other than DNA may well influence the sensitivity of the tumour towards alkylating agents. It has been shown for a small number of transplanted tumours growing in the BALB/C mouse that an apparent relationship exists between the ratio protein bound SH/protein free SH and sensitivity to a nitrogen mustard [33]. An increased intracellular concentration of a non-essential nucleophile very reactive towards alkylating agents may be of particular importance in determining the sensitivity of tumours showing acquired resistance to alkylating agents. The resistant line is usually remarkably similar to the present line in its growth characteristics and biochemical and morphological features [34, 35], yet it may be as much as 2000 times less sensitive to alkylating agents. In studies on the Yoshida sarcoma and a resistant line derived from it, two individual groups of workers have reported that the resistant line has a higher content of protein free thiol groups [34, 36]. However, since this is glutathione which is not a particularly reactive nucleophile towards alkylating agents, it is difficult to see how the increased concentration reported for the thiol could account for the large degree of resistance. The resistant line would not necessarily need to show a high increased intracellular concentration of a nucleophile, but merely a high concentration in the proximity of the DNA, and in a preliminary observation, the nuclei of resistant Yoshida sarcoma cells have been shown to have a higher content of SH groups [37] than the cells of the parent sensitive line. In this connec-

tion it is of interest that Jellum and Eldjarn [38] have shown that certain histones have a high content of reactive thiol groups. The high content of thiol groups of the nucleus of the resistant Yoshida tumour may be an indication that it has histones particularly rich in thiols. If this is found to be the case one may see where two such tumours identical in most respects may nevertheless show an enormous difference in sensitivity. The alkylating agents probably inactivate DNA by cross linking guanine bases [11]. DNA is so sensitive to this alkylation, that reaction need take place with only one in 10,000 nucleotides before complete inactivation of DNA, and cytotoxicity occurs. The resistant tumour might contain thiol rich histones so situated that a thiol group is adjacent to every guanine base. This thiol group might be very reactive towards alkylating agents so that carbonium ions formed close to the guanine bases react preferentially with the thiol rather than with the guanine. It would require a second carbonium ion to form close to a guanine before cytotoxic DNA alkylation took place. Since at toxic doses the level of DNA alkylation is extremely low only a small number of available guanine bases are actually alkylated. If each guanine were protected from the first alkylation by a histone thiol, the chances of it being attacked by a second ion would be extremely small. In order to get a comparable level of DNA alkylation in such a situation, the concentration of alkylating agent would need to be very much higher.

RÉSUMÉ

Bien que les thiols soient capables d'exercer une protection contre la toxicité provoquée par les rayons X et par les agents alkylants, il semble bien que le mécanisme protecteur ne soit pas identique dans les deux cas. Tous les composés qui sont capables d'exercer une bonne protection contre les agents alkylants atteignent une haute concentration sous forme libre, chez l'animal, et sont très réactifs à l'égard des agents alkylants. Il est vraisemblable que ces composés réagissent directement in vivo *avec les agents alkylants, et que cette réaction limite l'alkylation de l'ADN et explique ainsi leur action protectrice. L'application de la protection par les thiols à la chimiothérapie du cancer est de valeur douteuse, puisque, même au cas où des quantités protectrices de thiols pourraient être administrées, il est vraisemblable que la protection de l'hôte serait accompagnée d'une protection parallèle de la tumeur, sans améliorer l'action sélective de l'agent alkylant. La relation possible entre la teneur des tumeurs en thiol et leur résistance aux agents alkylants est discutée.*

SUMMARY

Although thiols may protect against both X-irradiation and alkylating agent induced toxicity there is evidence that the protective mechanism is not the same in both cases. All compounds that have so far been shown to give good protection against alkylating agents, attain a high concentration in free form in the animal and are very reactive towards alkylating agents. It is likely that they will react directly in vivo *with administered alkylating agents and such a reaction would restrict their alkylation of DNA and could thus account entirely for the protective effect. The application of thiol protection to cancer chemotherapy is of doubtful value since even if protective amounts of thiol could be administered protection to the host would be likely to be paralleled by protection to the tumour with no gain in selectivity of action of the alkylating agent. The possible relation between thiol content and resistance of tumours to alkylating agents has been discussed.*

ZUSAMMENFASSUNG

Obwohl die Thiolgruppen einen Schutz gegen die schädigende Wirkung sowohl der Röntgenstrahlen wie auch der alkylisierenden Mittel gewährleisten, scheint es sich in beiden Fällen um verschiedene Schutzmechanismen zu handeln.

Alle Verbindungen, welche den alkylisierenden Mitteln gegenüber diesen virusamen Schutz ausüben, erreichen in freier Form im tierischen Organismus eine hohe Konzentration und sind den alkylisierenden Mitteln gegenüber sehr reaktionsfähig.

Es ist wahrscheinlich, dass diese Verbindungen sofort in vivo *mit den verabreichten alkylisierenden Mitteln reagieren und dass durch diese Reaktion die Alkylierung der DNS*

gehemmt wird und so die Schutzwirkung erklärt werden kann. Die Zuwendung des Thiolschutzes im Falle der Chemotherapie des Krebses hat nur einen zweifelhaften Wert; da sogar im Falle einer Verabreichung von schutzfähigen Thiolgruppen ist es wahrscheinlich, dass der Schutz des Wirtes von einem gleichzeitigen Schutz des Tumors begleitet wird, ohne die selektive Wirkung des alkylisierenden Mittels zu verbessern.

Ein möglicher Zusammenhang zwischen dem Thiolgehalt der Tumoren und ihrer Resistenz den alkylisierenden Mitteln gegenüber wird diskutiert.

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Heterogeneity of Base Composition of DNA Extracted from Human Leukaemic Leucocytes

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IN A DOUBLE-STRANDED DNA molecule, according to the model of Watson and Crick, the four usual bases are placed in such a way that adenine and guanine are always paired, respectively, with thymine and cytosine of the complementary strand.

The ratio between the base pairs, adenine + thymine (A + T) and guanine + cytosine (G + C), is typical for each species of DNA, and the content of guanine + cytosine (G + C) is usually taken as an expression of the base composition of DNA.

Regarding its base composition, DNA has two essential features: the difference of the mean base compositions among different organisms and the heterogeneity of base composition of the DNA of a single species.

While lower organisms have a great variability among different species in the DNA base composition, the DNAs from different mammalian species generally show a similar overall base composition, their mean G + C content ranging from 40 to 44 per cent. However, the DNA of a single mammalian species displays, in general, a much higher degree of intermolecular heterogeneity of base composition than bacterial and viral DNA.

This has been shown particularly by CsCl density gradient centrifugation of the DNA in the analytical ultracentrifuge according to the technique of Meselson *et al.* [1]. A direct correlation has been established between density and G + C content of DNA [2], and the width of the band formed by DNA in CsCl has been correlated to the heterogeneity and the molecular weight of DNA.

Extensive studies of DNA by CsCl density gradient centrifugation indicated that in the majority of cases DNA forms a single band in the gradient [3]. In a few cases DNA can present satellite bands showing a density different from that of the main band. In mammalian species, satellite bands were observed in beef, mouse and guinea-pig tissue DNA, and their nature is at present unknown [4]. They could be formed by DNA having a different base composition or containing an unusual base or attached to other material of different density.

Another property of DNA, which has been found to depend on its base composition, is the temperature (*Tm*) at which DNA changes its double-stranded configuration to a random coil state, corresponding to an increase of optical density of the DNA solution at 260 mμ. The *Tm* of the DNA in solution increases with the increase of the G + C content of DNA [5]. Furthermore, the slope of the melting profile is indicative of the degree of heterogeneity of DNA.

DNA from different human tissues, analysed by CsCl gradient centrifugation, forms a single band with a mean density of 1·699 g/cm^3, corresponding to the usual G + C content of mammalian DNA in general. Also, DNA from human leukaemic leucocytes has a mean density similar to that of normal human DNA [6].

Another feature of the band formed in a CsCl gradient by mammalian DNA in general, and leukaemic DNA in particular, is its asymmetric shape. The band is skewed to the denser side. This asymmetry could be due to heterogeneity of base composition or to the presence

of some denatured DNA which displays an increased density in comparison to the corresponding native DNA due to the collapse of its structure.

In previous studies [6] it was shown that the human leukaemic DNA band in CsCl, particularly in the case of chronic myeloid leukaemia may undergo a considerable broadening and increase of the asymmetry when dissolved in low ionic strength solutions. This is probably due to denaturation of some DNA molecules. In the same conditions, normal human DNA does not undergo denaturation and its band in CsCl is not affected.

The hypothesis was raised that even the asymmetry present in the native leukaemic DNA band could be indicative of a collapsed structure, similar to that of denatured DNA, in some native DNA molecules.

In the present investigation, evidence is given in favour of the heterogeneity of base composition of leukaemic DNA, and by fractionation in a CsCl gradient in the preparative ultracentrifuge different DNA fractions have been isolated and their density and melting profiles have been studied.

METHODS

1. *Isolation of DNA*

Heparinized blood samples were obtained from patients affected by acute, lymphatic or myeloid leukaemia with high white blood cell counts. After allowing the heparinized blood to settle, the leucocyte-rich supernatant was pipetted off. The leucocytes were then collected by centrifugation and washed as previously reported [7]. DNA was extracted from the leucocytes by the method of Kay, Simmons and Dounce [8] or by the method of Kirby [9] or by the method of Marmur [10].

2. *Treatment with* Mustelus canis *liver deoxyribonuclease*

Leukaemic DNA was treated with a deoxyribonuclease extracted from *Mustelus canis* liver [11], which was shown to be active only on single-stranded denatured DNA. The reaction was carried out as reported. 0·1 ml of a 1% solution of the enzyme was added to 1 ml of SSC (0·15 M NaCl, 0·015 M Na citrate, pH 7) plus $1{\cdot}5 \times 10^{-3}$ M $MnCl_2$ containing 15 μg of native leukaemic DNA; the reaction mixture was then heated at 37°C for 60 min.

In a control experiment, heat-denatured leukaemic DNA was treated with the enzyme under the same experimental conditions.

3. *Renaturation of DNA*

The DNA was submitted to the optimum conditions used to reanneal denatured DNA [12]. DNA was heated at 65°C for 5 hr at a concentration of 10 μg/ml in 2 × SSC and then slowly cooled to room temperature. If some partially denatured DNA molecules were present, they should renature when submitted to this treatment.

4. *Analytical CsCl density-gradient centrifugation*

At the end of the enzymatic reaction and after the experiment of renaturation, the DNA was analysed in a CsCl density gradient in the analytical ultracentrifuge as described by Schildkraut *et al.* [2]; 0·89 g of optical grade CsCl, obtained from Merck AG., were added to 0·7 ml of SSC containing 2–4 μg of the DNA sample to be analysed and 0·5 μg of a marker DNA of known density. The density of the solution was adjusted to 1·710 g/cm³, and was checked by reading the refractive index.

The DNA solution was then centrifuged in the analytical ultracentrifuge cell at 44,700 rev/min at 25°C for 20 hr. At the end of the centrifugation period ultraviolet photographs were taken on Kodak commercial films. The photographs were scanned by a microdensitometer and the densities of the DNA bands were calculated from their position in relation to the marker DNA using the formula reported by Schildkraut *et al.* [2].

5. *Determination of the melting temperature*

The absorbance-temperature profile of the DNA samples was determined as reported by Marmur and Doty [5].

DNA in solution in SSC at a concentration of 20 μg/ml was heated in glass-stoppered quartz cuvettes in a Beckman DU spectrophotometer equipped with thermal spacers for circulating hot liquid and a thermometer. While increasing the temperature from 25°C to 100°C, the optical density was read every 2 degrees. The values of optical density obtained were plotted against the temperature. The melting temperature (*Tm*) is the temperature corresponding to 50 per cent of the increase in optical density.

6. *Fractionation of DNA by CsCl preparative ultracentrifugation*

Leukaemic DNA was fractionated by means of the CsCl gradient preparative centrifugation following essentially the technique described by Szybalsky [13].

A solution of DNA at a concentration of 20 μg/ml in SSC containing CsCl (density = 1·71 g/cm³) was centrifuged in 5-ml lusteroid

tubes in the SW 39 swinging bucket rotor of the Spinco L_1 preparative ultracentrifuge at 33,000 rev/min for 72 hr. At the end of the centrifugation period the bottom of the tube was punctured by a needle and two-drop fractions (volume 0·09 ml each) were collected separately.

The heavier fractions of the DNA band were pooled together and further purified by fractionation in a second CsCl gradient.

The density and the melting temperature of the purified DNA fractions were determined as cited above.

Their denaturation was carried out by heating at 100°C for 10 min in SSC or 1/10 SSC, and their renaturation was attempted by the technique described above.

RESULTS

When native DNA extracted from leukaemic leucocytes is banded in a CsCl gradient in the analytical ultracentrifuge (Fig. 3a) a band similar to that formed by human normal leucocytes DNA is obtained, as previously reported [6].

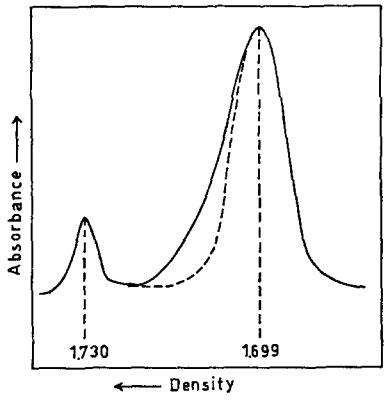

Fig. 1. Densitometer tracing of DNA extracted from chronic myeloid leukaemia leucocytes, at equilibrium in a CsCl density gradient in the analytical ultracentrifuge. The DNA had been treated with Mustelus canis *liver deoxyribonuclease prior to centrifugation. The DNA solution containing CsCl (density= 1·71 g/cm³) was centrifuged at* 44,700 *rev/min for* 20 *hr in the analytical ultracentrifuge. The peak on the left corresponds to* Streptomyces albus *DNA (density=1·730 g/cm³) which was added as a density marker.*

The result obtained by treating DNA extracted from chronic myeloid leukaemic leucocytes with *Mustelus canis* liver deoxyribonuclease is illustrated in Fig. 1. The band formed in a CsCl density gradient by the DNA treated with such enzyme is asymmetric. The extent of the asymmetry is the same as when the untreated DNA was banded in the gradient.

Similar results were obtained when the DNA was heated at 65°C for 5 hr to favour the renaturation of denatured molecules prior to centrifugation; no difference was noted when compared to the unheated DNA.

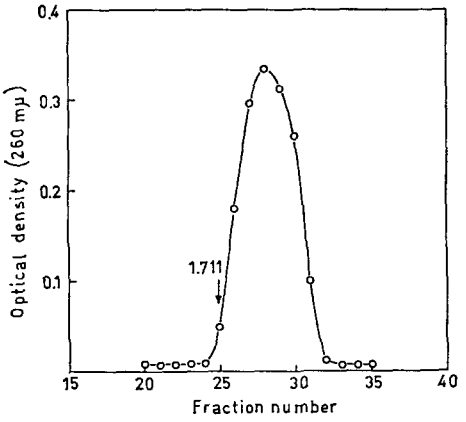

Fig. 2. Fractionation of DNA extracted from chronic myeloid leukaemia leucocytes by CsCl preparative ultracentrifugation. The position of the densest fraction (density=1·711 g/cm³) is indicated by an arrow.

Also, the shape of the bands formed by DNA from acute and lymphatic leukaemia leucocytes was not affected by the above-cited treatments.

By means of the fractionation of the DNA of leukaemic leucocytes by CsCl density gradient preparative centrifugation, eight DNA fractions were separated at different levels in the region of the CsCl gradient containing DNA.

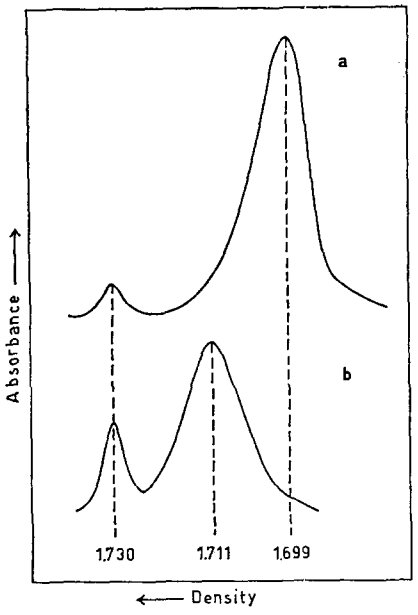

Fig. 3. Microdensitometer tracings of unfractionated leukaemic DNA (a) and of the densest fraction (b) isolated from the same DNA sample by the fractionation experiment reported in Fig. 2. The DNA fraction(b) was obtained by pooling the heavy DNA fractions collected from several tubes and purifying them in a second CsCl gradient. The equilibrium in the gradient had been reached by centrifuging at 44,700 *rev/min for* 20 *hr. The peak on the left corresponds to* Streptomyces albus *DNA.*

The results obtained in a fractionation experiment are illustrated in Fig. 2. The fractions of the DNA band corresponding to the peak and to the less dense side, when analysed in the analytical ultracentrifuge, showed a density close to the mean density of the unfractionated sample. A fraction displaying a density higher than the mean density of the band was isolated from the denser side.

Each of the DNA fractions, when analysed in a CsCl gradient in the analytical centrifuge after heat denaturation, showed an increase in density similar to that of a normal double-stranded DNA.

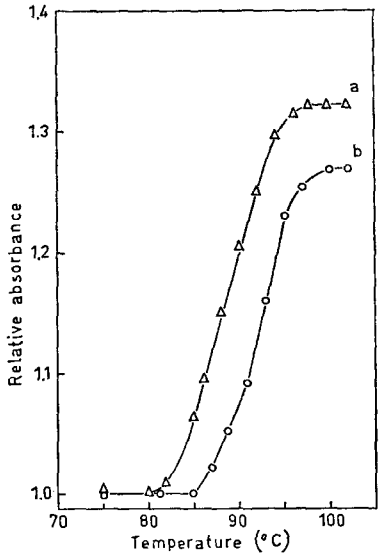

Fig. 4. Absorbance temperature curves of DNA from human leukaemic leucocytes: (a) unfractionated DNA (b) DNA fraction of the denser side of the whole DNA band, isolated by CsCl density gradient preparative centrifugation, corresponding to (b) of Fig. 3.

The densest fraction in our experiments had a density of 1·711 g/cm³ (Fig. 3). An attempt to reanneal such a purified DNA fraction after heat denaturation was not successful, as shown by its density which did not return to the value of native DNA when exposed to conditions favouring renaturation.

The melting temperatures (*Tm*) of the DNA fractions were in agreement with their buoyant densities, according to the relations reported by Marmur and Doty [4] and Schildkraut *et al.* [2]. In Fig. 4 the melting profile of the DNA fraction displaying a density of 1·711 g/cm³ is reported together with the melting profile of the unfractionated DNA sample.

DISCUSSION

The present results indicate that the asymmetry of the band formed in density gradient by native DNA extracted from human leukaemic leucocytes is due to heterogeneity of base composition.

This is shown by the persistence of the asymmetry after treatments (renaturation, digestion with dogfish liver deoxyribonuclease) upon which denatured or partially denatured DNA molecules should disappear.

This is also supported by the isolation from the bulk of leukaemic DNA of a DNA fraction which displays a higher density (1·711 g/cm³), increases in density like native double-stranded DNA upon denaturation, and displays an absorbance–temperature curve with a *Tm* in agreement with its buoyant density.

Neither such a fraction nor other DNA fractions isolated by the fractionation procedure used are able to renature, in such way behaving like all normal mammalian DNA, the only exceptions being mitochondrial DNA and calf thymus satellite DNA which are able to renature, as recently shown [14–16].

It is to be noted that the extent of the asymmetry of the DNA band seems to vary in different samples of leukemic DNA, even though the same experimental procedures are used to extract DNA. This is particularly true in the case of DNA extracted from leucocytes of chronic myeloid leukaemia.

The asymmetry of the band in CsCl is not a unique property of DNA extracted from leukaemic cells but is present to some extent also in all normal mammalian DNA.

In a previous paper [6] it was shown that the band formed in CsCl by DNA extracted from human leukaemic leucocytes, particularly in the case of chronic myeloid leukaemia, undergoes a broadening and increase of the asymmetry when the DNA is dissolved in solution at low ionic strength. This is probably due to denaturation of some of the DNA molecules. The hypothesis was raised that a fraction of denatured DNA in a random coil state could also be responsible for the asymmetry of the band of native leukaemic DNA.

The present results do not support this hypothesis as the fraction isolated from the denser side of the native leukaemic DNA band is double-stranded DNA.

The variability in broadness and asymmetry of the band of human leukaemic DNA in CsCl might well be the result of a different extent of breaking of the DNA molecules by the shearing forces of the extraction procedure, but it is at present dubious whether this is indicative of a greater intrinsic lability of leukaemic DNA, or is due to the presence of more active DNAase in

leukaemic cells, or to other secondary unknown reasons.

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RÉSUMÉ

L'ADN, extrait des leucocytes leucémiques humains, forme une bande (densité=1,699 *g/cm³) asymétrique du côté plus dense dans un gradient de CsCl; l'état d'équilibre est réalisé dans l'ultracentrifugeuse analytique.*

Cette assymétrie est présente, a un certain degré également, dans les ADN normaux de mammifère. Elle est due à l'hétérogénéité de composition en bases de l'ADN, comme le montre sa persistence après des traitements (renaturation, digestion avec désoxyribonucléase du foie de Mustelus canis*) qui devraient faire disparaître l'asymétrie, si elle était due à des molécules d'ADN dénaturé.*

Au moyen de l'ultracentrifugation préparative en CsCl, une fraction d'ADN, d'une densité de 1·711 *g/cm³, a été isolée du côté plus dense de la bande de l'ADN leucémique. Cette fraction est formée par l'ADN natif "double-stranded", comme le montre l'augmentation de la densité optique après dénaturation thermique.*

L'importance de l'asymétrie de la bande d'ADN semble être plus variable dans l'ADN leucémique (particulièrement dans le cas de la leucémie myéloïde chronique) que dans l'ADN normal de mammifère. L'interprétation de ces résultats est discutée.

SUMMARY

The DNA extracted from human leukaemic leucocytes forms a band (density=1·699 *g/cm³) asymmetric to the denser side in a CsCl gradient at equilibrium in the analytical ultracentrifuge. Such asymmetry is present to some extent also in every normal mammalian DNA. It is due to heterogeneity of DNA base composition, as shown by its persistence after treatments (renaturation, digestion with* Mustelus canis *liver deoxyribonuclease) upon which the asymmetry should disappear if it were due to denatured DNA molecules.*

By CsCl preparative centrifugation of leukaemic DNA, a DNA fraction displaying a density of 1·711 *g/cm³ has been isolated from the denser side of the band. This fraction is formed by native double-stranded DNA, as shown by the density shift and the increase in optical density upon thermal denaturation.*

The extent of asymmetry of DNA band seems to be greater and more variable in leukaemic DNA (particularly in the case of chronic myeloid leukaemia) than in normal mammalian DNA. The interpretation of this finding is discussed.

ZUSAMMENFASSUNG

Die aus menschlichen leukämischen Leukozyten extrahierte DNS, bildet eine auf der dichteren Seite asymmetrische Zone (Dichte=1,699 *g/cm³) in einem CsCl-Gradienten in der analytischen Ultrazentrifuge. Eine solche Asymmetrie findet man in gewissem Umfang ebenfalls in jeder normalen Säugetier-DNS. Sie ist der Heterogenität der Basenzusammensetzung der DNS zuzuschreiben, was durch ihr Fortbestehen nach Behandlung (Renaturation, Verdauung mit* Mustelus canis *Leber-Desoxyribenuclease) gezeigt wird, nach welcher die Asymmetrie verschwinden müsste, wenn sie auf denaturierte DNS-Moleküle zurückzuführen wäre.*

Durch präparative CsCl-Zentrifugation von leukämischer DNS, konnte eine DNS-Fraktion mit einer Dichte von 1,711 *g/cm³ auf der dichteren Seite der Zone isoliert werden. Diese Fraktion setzt sich aus einer nativen "double-stranded" DNS zusammen, was durch den Dichteunterschied und das Ansteigen der optischen Dichte nach Wärmedenaturation gezeigt wird. Die Grösse der Assymmetrie der DNS-Zone scheint in leukämischer DNS (vor allem im Fall von chronischer myeloischer Leukämie) beträchtlicher und veränderlicher zu sein, als in normaler Säugetier-DNS. Die Erläuterung dieser Befunde wird diskutiert.*

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An Investigation into Thecomatosis of the Ovary and Related Tumours by Histochemical and Enzyme Histochemical Methods

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INTRODUCTION

HYPERTROPHY of the theca cells during atresia of ovarian follicles is a well-known fact and in pregnancy it is sometimes very pronounced. In some pathological circumstances the number of theca cells in the ovaries of neonates [1], children [2] and women before and after the menopause can be increased [3]. In the ovaries of post-menopausal women particularly, masses of dense, well vascularized, stromal tissue with a tendency to whorl formation may develop in either the cortex or the medulla. This is called cortical stromal hyperplasia. Sometimes some of these cells become large and polyhedral and contain fat, thus resembling cells of the theca interna. This phenomenon is described as thecomatosis [4], thecosis or hyperthecosis [5]. Both cortical stromal hyperplasia and this form of thecomatosis are in some way related to the development of carcinoma of the endometrium [6] and it has been suggested that these abnormal ovarian structures produce oestrogens which may play a part in the development of this tumour. However, Charles *et al.* [7] did not find an increased output of oestrogens in the urine of patients with carcinoma of the endometrium notwithstanding the fact that six of nine patients had thecomatosis of the ovaries. *In vitro* work, with labelled steroid substrates, has confirmed that stromal cells can produce oestrogens independently from the luteofollicular apparatus [8]. It is, however, very difficult to localize by biochemical methods the cells responsible for steroidogenesis in an organ that contains different structures, such as the human ovary and ovarian tumours. It has not so far been possible to make a sharp distinction between steroid-producing and non-steroid-producing tumours. Besides steroid-producing tumour cells, it is likely that some tumours in the ovary induce the stroma to produce steroids [9]. In recent years some enzyme histochemical methods, especially those for the demonstration of the enzymes 3β-ol-hydroxysteroid dehydrogenase [10–12] and secondary alcohol dehydrogenase [12], have been developed to study this problem. But, in contrast to biochemical methods, not all details of steroidogenesis, e.g. aromatization of the A-ring, can be demonstrated. Thus both methods, biochemical and histochemical, must be used to study the problems of normal and pathological processes in steroid-producing organs.

We have attempted to contribute to the solution of this problem by studying by histochemical and enzyme histochemical methods

the localization of steroidogenesis in the normal ovary, in ovarian abnormalities related to the development of the carcinoma of the endometrium and in some ovarian tumours.

MATERIALS AND METHODS

Clinical and pathological data

Ovaries were obtained from surgical specimens. The presence or absence of primary follicles, growing follicles, atretic follicles, old atretic follicles, corpora albicantia, thecomatosis (including cortical stromal hyperplasia), thecoma and fibroma were recorded. Whenever possible the endometrium was examined also. The findings with some clinical data are summarized in Table 1. None of the patients had shown features of the Stein-Leventhal syndrome. In patients 15 and 16 the ovarian abnormalities were of particular importance. In each ovary of patient 15 there was typical thecomatosis and hyperplasia of hilus cells (Fig. 1). The large number of theca cells in the stroma was remarkable (Fig. 2). A tumour in the right ovary was composed of polyhedral and fusiform cells (Fig. 3). Adjacent to and within the tumour there were some cells that resembled theca cells (Fig. 4). The morphological differences between these cells and the tumour cells were not so clear as in thecomatosis. Patient 16 had a large greyish ovarian tumour with

Table 1. *Clinical and pathological data*

Patient No.	age	Ovaries									Endometrium	Reason for operation (one autopsy)
		Primary follicles	Growing follicles	Atretic follicles	Old atretic follicles	Corpus luteum	Corpus albicans	Thecomatosis	Thecoma	Fibroma		
1.	42	+	+	+	+	+	+	−	−	−	Adenocarcinoma	Adenocarcinoma of corpus uteri
2.	40	+	+	+	+	+	+	−	−	−	Proliferating	Carcinoma *in situ* of cervix uteri
3.	38	+	+	+	+	+	+	−	−	−	Unknown	Menorrhagia and enlarged tender adnexa uteri
4.	37	+	+	+	+	−	+	−	−	−	Proliferating	Irregular uterine bleeding
5.	37	−	−	−	+	−	+	−	−	−	Unknown	Squamous cell cancer of cervix uteri
6.	68	−	−	−	+	−	+	−	−	−	Unknown	Multilocular serous cystadenoma
7.	44	+	+	+	+	−	+	(+)	−	−	Unknown	Uterine myomas and irregular uterine bleeding
8.	53	−	−	+	+	−	−	(+)	−	−	Hyperplastic	Uterine myomas and irregular uterine bleeding
9.	72	−	−	−	−	−	+	(+++)	−	−	Proliferating	Autopsy
10.	51	−	+	+	+	−	+	++/+	−	−	Proliferating	Uterine myomas and irregular uterine bleeding
11.	60	−	−	−	+	−	+	++/+	−	−	Active stroma with atrophic cystic tubules	Menopause at the age of 35; irregular uterine bleeding from the age of 50
12.	51	−	−	−	+	−	+	++	−	−	Proliferating	Irregular uterine bleeding
13.	78	−	−	−	+	−	+	+++/++	−	−	Hypoplastic	Ovarian cystadenocarcinoma
14.	72	−	−	−	+	−	+	+++	−	−	Hyperplastic	Rö-castration at the age of 46 followed by irregular uterine bleeding
15.	75	−	−	−	−	−	+	+++	+	−	Adenocarcinoma	Adenocarcinoma of corpus uteri
16.	54	No ovarian tissue left								+	Hypoplastic	Large ovarian fibroma

( ) Means hyperplastic stromal islands without luteinized cells.

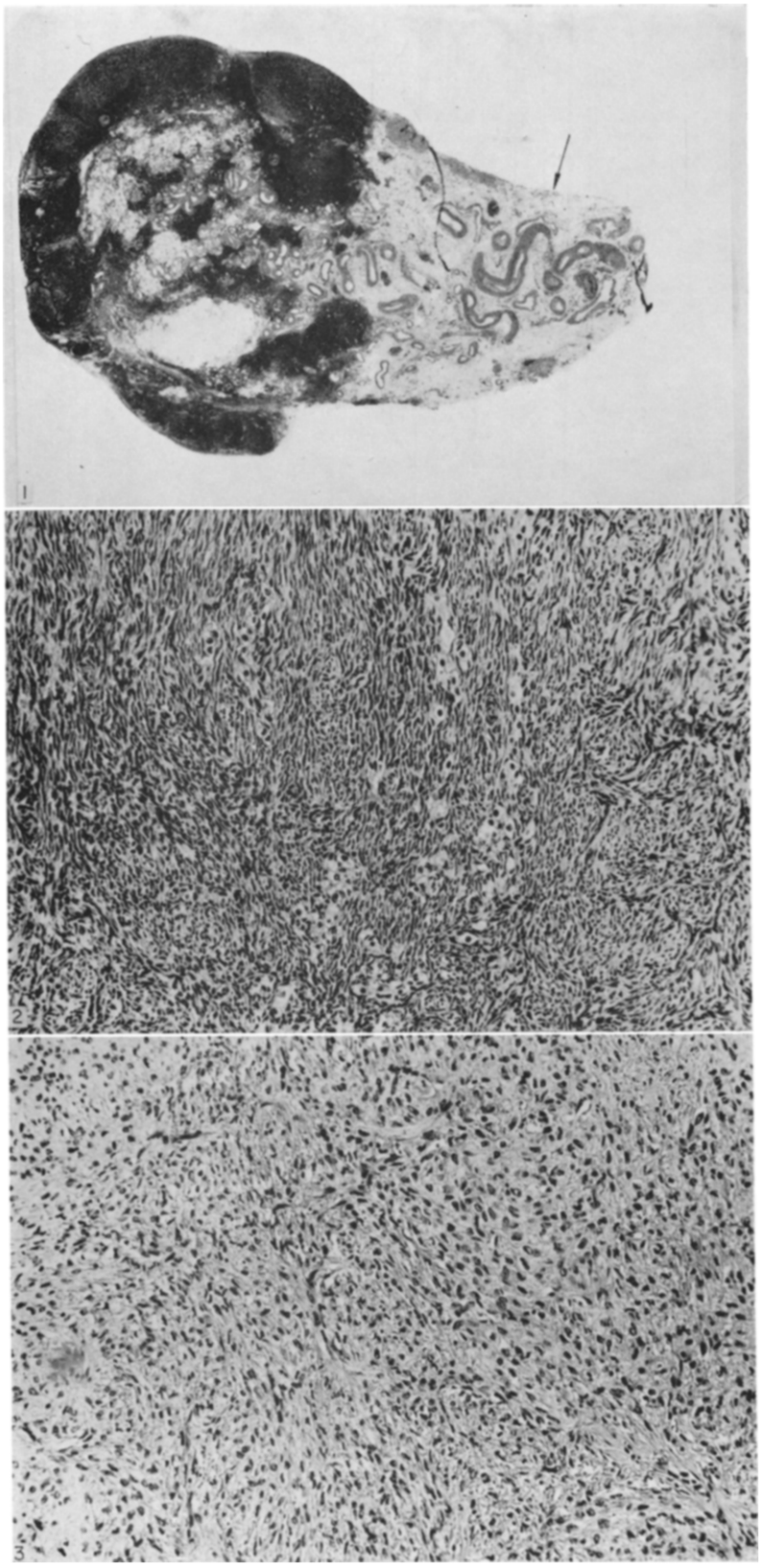

Fig. 1. *Cryostat section. Haematoxylin eosin stain.* ×5. *Section of the left ovary of patient* 15. *Foci of cortical stromal hyperplasia in the cortex and medulla. Groups of epitheloid cells can be seen in the hilus (arrow).*

Fig. 2. *Paraffin section. Haematoxylin eosin stain.* ×120. *Part of the hyperplastic stroma in Fig.* 1. *Epitheloid cells with clear cytoplasm and round nuclei (theca cells) are seen (between the fibroblasts). This is one type of thecomatosis.*

Fig. 3. *Paraffin section. Haematoxylin eosin stain.* ×120. *Thecoma of patient* 15. *The tumour is composed of large cells, rather like fibroblasts, which form whorls.*

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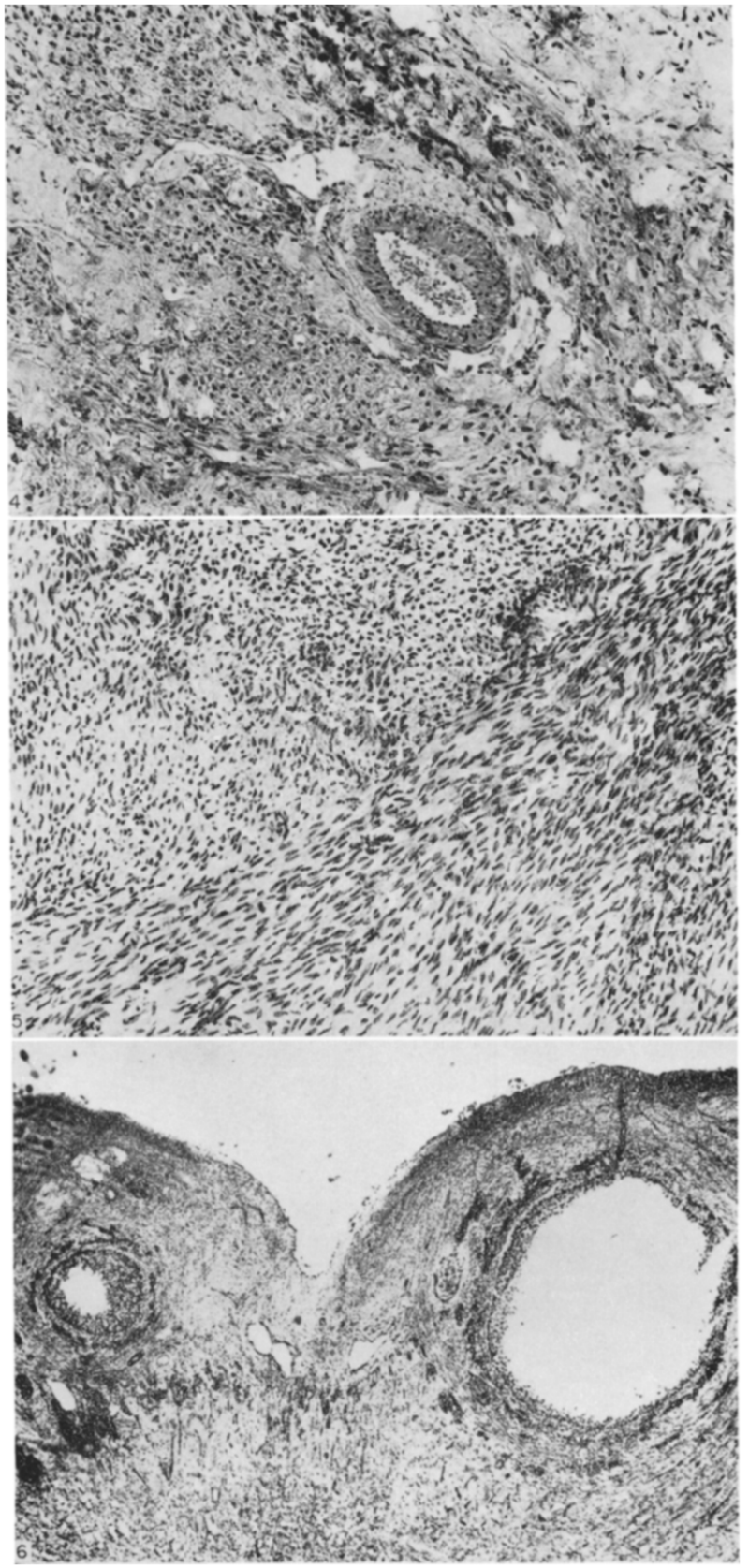

Fig. 4. *Paraffin section. Haematoxylin eosin stain.* ×120. *Another part of the thecoma shown in Fig.* 3. *Small groups of epitheloid cells are seen, especially on the outside of the tumour. They resemble the theca cells of the normal ovary. The morphological difference between the thecal stromal cells and these "luteinized" cells is not sharp.*

Fig. 5. *Paraffin section. Haematoxylin eosin stain.* ×120. *Fibroma of patient* 16. *The tumour is composed of small elongated fibroblasts.*

Fig. 6. *Cryostat section. Reduced NAD-tetrazolium reductase.* ×50. *Growing follicles, and inferiorly theca cells of an atretic follicle. Theca cells show strong enzyme activity.*

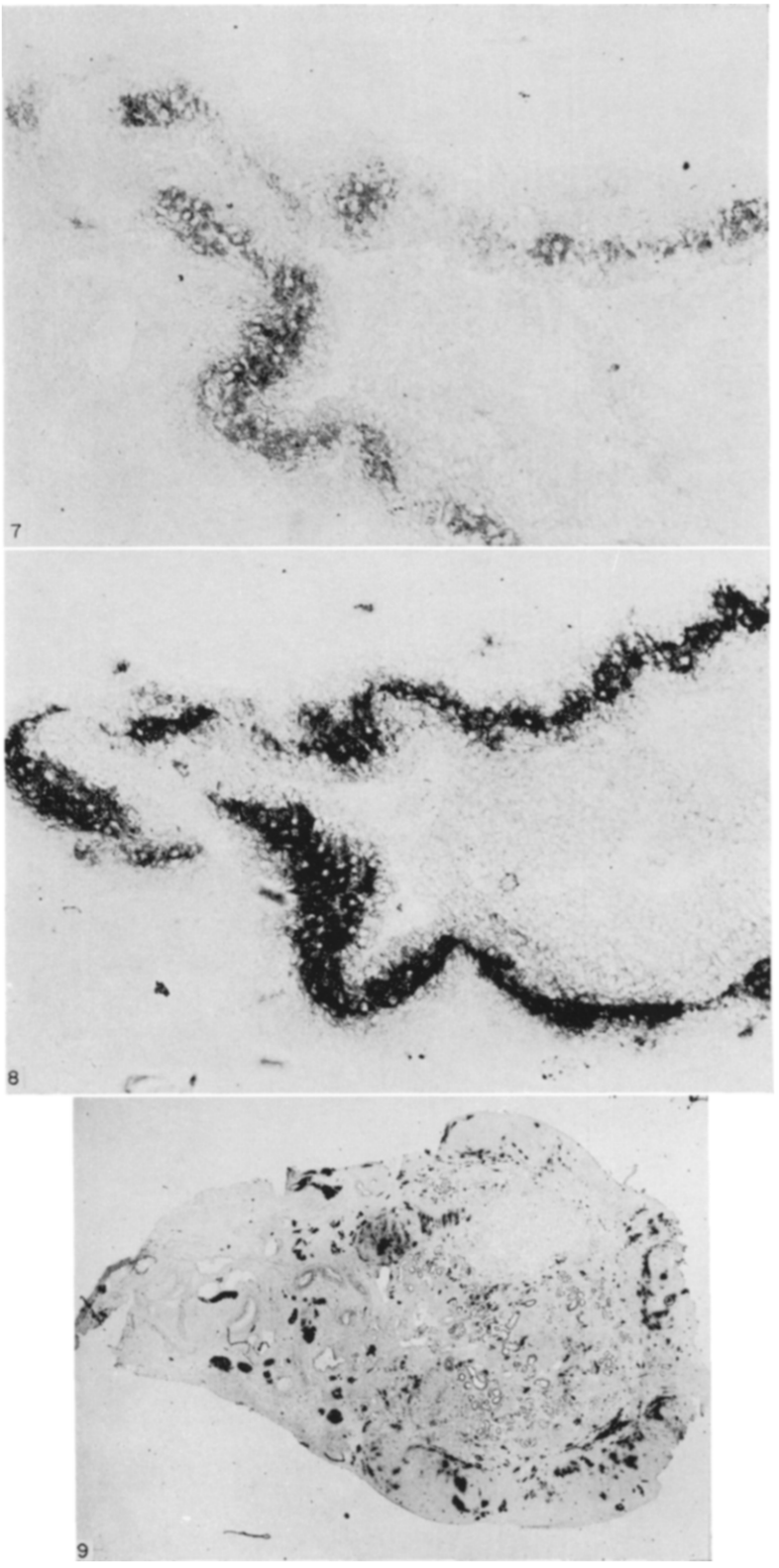

Fig. 7. *Cryostat section.* 3β-*ol Hydrosteroid dehydrogenase. Allen's method using dehydroepiandrosteron as substrate.* ×120. *Growing follicles. Theca cells show activity.*

Fig. 8. *Cryostat section.* 3β-*ol Hydroxysteroid dehydrogenase. Allen's method using androstenediol as substrate.* ×120. *The same follicle as in Fig.* 7. *With this substrate the activity is very strong. Control section without substrate was negative.*

Fig. 9. *Cryostat section. Secondary alcohol dehydrogenase.* ×5. *The same ovary as Fig.* 1. *Theca cells of thecomatosis and ovarian hilus cells show very strong enzyme activity.*

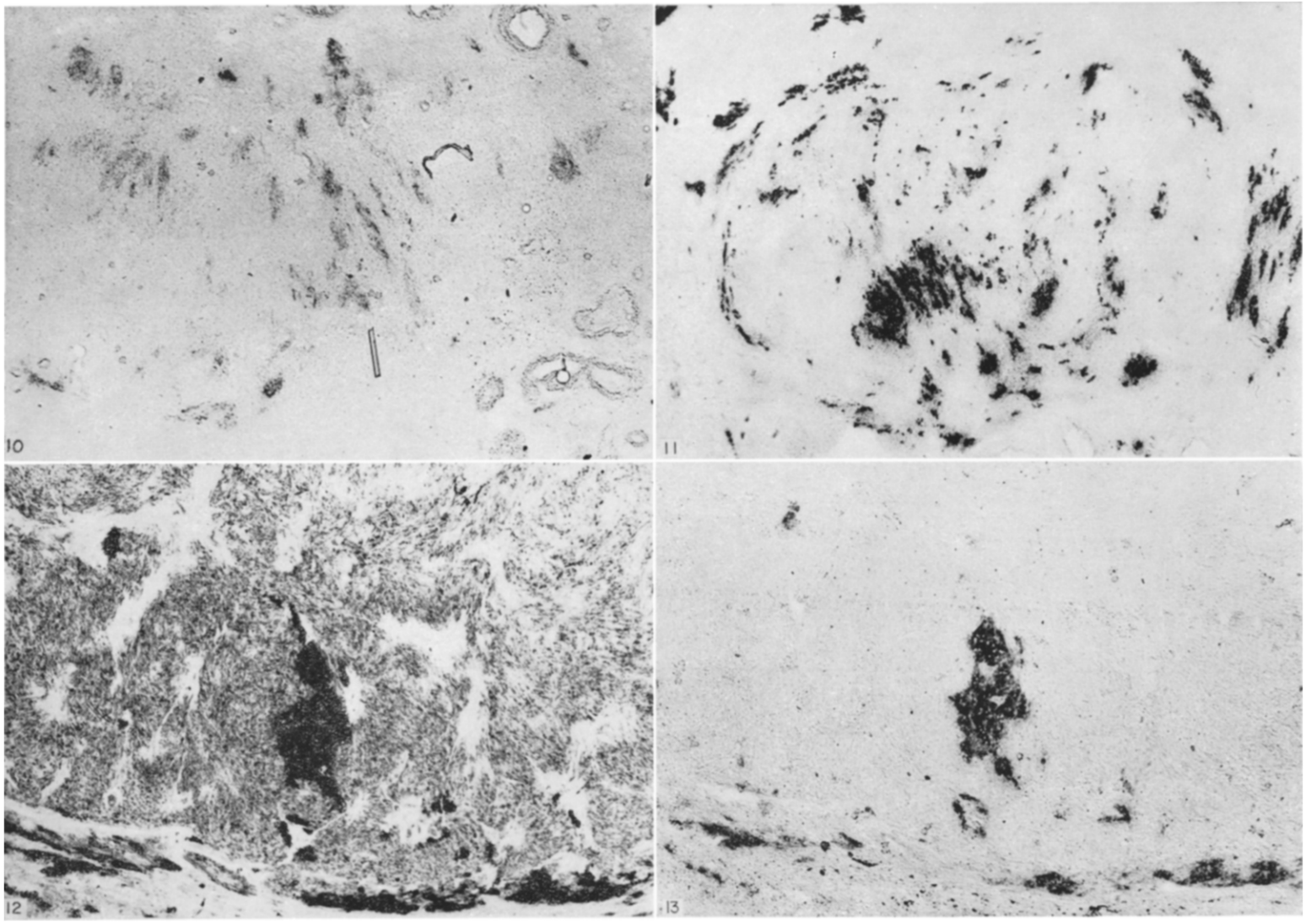

Fig. 10. *Cryostat section.* 3β-*ol Hydroxysteroid dehydrogenase. Allen's method using androstenediol substrate.* ×50. *The theca cells of the thecomatosis in patient* 15 *show moderate activity. Control section without subtrate was totally negative.*

Fig. 11. *Cryostat section. Secondary alcohol dehydrogenase.* ×50. *The same area as Fig.* 10. *The theca cells show very strong enzyme activity.*

Fig. 12. *Cryostat section. Glusoce-*6*-phosphate dehydrogenase.* ×50. *Thecoma of patient* 15. *The thecal stroma cells show moderate activity, but groups of cells on the outside and within the tumour show very strong enzyme activity.*

Fig. 13. *Cryostat section. Secondary alcohol dehydrogenase.* ×50. *The same area as Fig.* 12. *Only the luteinized cells show strong enzyme activity.*

yellowish foci and a rubbery consistency. Microscopically the tumour, which was composed of fibroblasts (Fig. 5), was diagnosed as a fibroma.

Staining reactions

Parts of the surgically excised ovaries were frozen with CO_2 within half an hour of removal. Serial sections 10μ thick were cut in a cryostat and stored at −25°C. The remaining ovarian tissue was embedded in paraffin wax.

The cryostat sections were stained with haematoxylin and eosin, Oil red O, Sudan Black B and the periodic acid Schiff reaction (PAS). The following enzyme reactions were performed: alkaline phosphatase by both the metal salt [14] and azo-dye techniques [15]; acid phosphatase [14]; adenosine triphosphatase [15]; 5-nucleotidase [16]; α-naphthyl-acetate esterase [17]; naphthol AS-D acetate esterase [18], 4 chloro-5-bromoindoxyl acetate esterase [19]; reduced nicotinamide-adenine-dinucleotide ($NADH_2$) tetrazolium reductase [20]; lactate dehydrogenase [21]; β-hydroxy-butyrate dehydrogenase [21]; reduced nicotin amide-adenine-dinucleotide phosphate ($NADPH_2$) tetrazolium reductase [20]; glucose-6-phosphate dehydrogenase [22], succinate dehydrogenase [23] with the addition of 0·1 mg phenazinemethosulphate per ml incubating medium [20]; α-glycerophosphate dehydrogenase with the addition of menadion [24]; 3β-ol hydroxysteroid dehydrogenase [12] both with dehydroepiandrosterone and Δ4-androstene-3β-17β-diol as substrate; 3β-ol hydroxysteroid dehydrogenase [11] with pregnenolone; dehydroepiandrosterone and Δ4-androstene-3β-17β-diol as substrates; secondary alcohol dehydrogenase [13] with isopropanol as substrate; alcohol dehydrogenase [13] with propanol as substrate.

In the phosphatase and esterase methods the sections were fixed in a mixture of 10 ml formaldehyde (40%), 90 ml dextran (6%) and 1 g calcium chloride for 10 min. After washing, the staining reactions were carried out according to the methods described above.

Prior to incubation the sections for the dehydrogenase methods were placed in acetone at −20°C for 30 min in order to dissolve lipid droplets, thus preventing adsorption of formazan to these droplets. The sections were then dried in air and incubated for 45 min at 37°C in the incubating media. After staining, the sections were fixed in the formalindextran mixture and embedded in glycerol gelatine. In the Allen method [12] for 3β-ol hydroxysteroid dehydrogenase the incubation was carried out at 37°C for 4 hr; in the Levy method [11] the 4-hr incubation was performed at 20°C. The incubation time for secondary alcohol dehydrogenase and alcohol dehydrogenase was 2 hr at 37°C.

RESULTS

1. *The luteo-follicular apparatus*

The results are summarized in Table 2. In the growing and atretic follicles the theca cells possessed the same enzyme pattern, although the activity of most enzymes was more or less decreased in the theca cells of the atretic follicles. In addition to lipids we found enzyme activity for alkaline and acid phosphatase, aminopeptidase, esterases, the tetrazolium reductases (Fig. 6), 3β-ol hydroxysteroid dehydrogenase (Figs. 7 and 8), glucose-6-phosphate dehydrogenase and secondary alcohol dehydrogenase. There was little or no activity of these enzymes in the granulosa cells of the follicles. The distribution of α-glycerophosphate dehydrogenase was remarkable as the strongest activity was registered in the granulosa cells. After rupture of the follicle great changes in the enzyme pattern appeared. Now the strongest enzyme activity for acid phosphatase, non-specific esterases, 3β-ol hydroxysteroid dehydrogenase and alcohol dehydrogenase was found in the granulosa cells, while that for alkaline phosphatase, the tetrazolium reductases, secondary alcohol dehydrogenase, glucose-6-phosphate dehydrogenase and aminopeptidase was found in the theca cells of the corpus luteum.

2. *Hilus cells*

Hyperplastic hilus cells were found only in the ovaries of patient 14, but by enzyme histochemical methods a few hilus cells were detected in some other ovaries. Apart from the absence of aminopeptidase they showed the same enzyme pattern as the theca cells of the growing follicles. The activities of acid phosphatase, naphthol AS-D esterase, glucose-6-phosphate dehydrogenase and secondary alcohol dehydrogenase (Fig. 9) were particularly strong. There was moderate activity of 3-β-ol hydroxysteroid dehydrogenase.

3. *Cortical stromal hyperplasia and thecomatosis*

In every case of cortical stromal hyperplasia we found varying numbers of cells that contained lipids and some enzymes, especially glucose-6-phosphate dehydrogenase, with a pattern similar to that found in the theca cells of growing and atretic follicles.

The enzyme activity of 3β-ol hydroxysteroid

Table 2. Enzyme pattern of ovarian follicles, corpus luteum of menstruation, thecomatosis, thecoma and fibroma of the human ovary

	Growing follicles		Atretic follicles		Corpus luteum menstr.		Thecomatosis		Thecoma		Fibroma
	T	G	T	G	T	G	L.C.	H.S.	L.C.	T.S	
Oil Red O	+/±	−	+	−	+++/++	++/+	+/±	±/−	+/±	++/+	+/−
Sudan Black B	+/±	−	+	−	++/+	+/−	+/±	±/−	+/±	++/+	+/−
Alkaline phosphatase	++	−	++	−	++/+	+/−	++/+	−	++/+	+/−	−
Acid phosphatase	++	+	++	±	++/+	+++	++	±	++	+/−	+/−
ATP-ase	±/−	−	±/−	−	−	−	−	−	−	−	
5-Nucleotidase	±/−	−	−	−	−	±/−	?	++	?	+/−	+/−
Non-specific esterases:											
α-Naphthyl acetate esterase	++/+	−	++/+	−	+/−	+++	+	−	+	−	−
Naphthol AS-D acetate esterase	±	−	+/−	−	±/−	++/+	±	−	±	−	−
Indoxyl acetate esterase	±	−	±	−	+/−	+++	±	−	±	−	−
NADH-tetraz. reductase	+++	++	+++/+	+	+++	++	+++	++	+++	+	+/−
Lactic acid dehydrogenase	++	+	++/−	+/−	++	++/+	++	+	++	+/±	±/−
β-Hydroxybutyric acid dehydr.	++	+	++/−	−	++/+	+	++	±/−	++	−	−
3β-ol Hydroxysteroid dehydr.											
Dehydroepiandrosterone (Allen)	±/−	−	±/−	−	+	+++	±/−	−	±/−	−	−
4-Androstene-3β-ol, 17β-ol (Allen)	+	−	±/−	−	±	+++	+	−	++	−	−
Pregnenolone (Levy a.o.)	±/−	−	±/−	−	±/−	++	±/−	−	±/−	−	−
Dehydroepiandrosterone (Levy a.o.)	±/−	−	±/−	−	±/−	++	±/−	−	±/−	−	−
4-Androstene-3β-ol, 17β-ol (Levy a.o.)	+	−	±/−	−	±	++	+	−	+	−	−
Second. alcohol dehydr.	+++	−	+++/+	−	+++	+	+++	−	+++	−	−
Alcohol dehydrogenase	+	±	+/−	−	+	+++	±	+	+	±/−	±/−
NADPH-tetrazolium reduct.	+++	++	+++/+	+/−	+++	++	+++	++	+++	+	+
Glucose-6-phosphate dehydr.	+++	+	+++/+	±/−	+++	++/+	+++	+	+++	+/±	±/−
Succinic dehydrogenase	++/+	+	++	+/−	++/+	+	++	+	++	±	+/−
α-Glycerophosphate oxidase	±/−	++	±	+/−	±	++/+	+/±	±	+/±	±/+	±/−
Aminopeptidase	++	−	+	−	++/+	−	−	−			−

T=theca cells; G=granulosa cells; L.C.=luteinised cells; H.S.=hyperplastic ovarian cortical stroma; T.S.=thecal stroma.
−, ±, +, ++, +++ mean respectively: negative, slight, moderate, strong and very strong enzyme activity; /=varying activity.

dehydrogenase in the epitheloid cells of thecomatosis seemed less than that in the theca cells of growing follicles (Fig. 10). No aminopeptidase activity was found. It appeared that we were dealing with varying grades of thecomatosis. The enzyme pattern of these structures are summarized in Table 2. In many cells of the hyperplastic cortical stroma, lipids and glucose-6-phosphate dehydrogenase were found. In addition to lipids the following enzymes related to steroidogenesis were identified in the luteinised cells of thecomatosis: 3β-ol hydroxysteroid dehydrogenase, secondary alcohol dehydrogenase (Fig. 11) and glucose-6-phosphate dehydrogenase. With these enzyme histochemical methods more cells with the characteristic enzyme pattern were found than would be expected from the examination of sections stained with haematoxylin and eosin.

It would appear, therefore, that luteinized cells can be more effectively identified by their enzyme pattern than by their morphological characteristics. Most cells were found with the enzyme glucose-6-phosphate dehydrogenase. In one patient (number 6) there was a lipid band in the cortex that did not contain cells with the enzyme pattern characteristic for steroidogenesis. Also no glucose-6-phosphate dehydrogenase could be found as in specialized cells of cortical stromal hyperplasia.

4. *Fibroma of the ovary*

The enzyme pattern of the fibroma of patient 16 is given in Table 2. In general it was the same as in the cells of the hyperplastic ovarian cortical stroma (Table 2). Many tumour cells contained lipids and a trace of acid phosphatase. No cells with the characteristics of theca cells were found.

5. *Thecoma of the ovary*

The enzyme pattern of the thecoma of patient 15 was obtained. The thecal stroma contained more lipid than the cells of the fibroma of patient 16. The activity of the enzyme acid phosphatase was also stronger. Groups of cells adjacent to and within the tumour showed the same enzyme pattern as the luteinized cells of thecomatosis (Figs. 12 and 13). It is evident that the composition of the thecoma resembles that of an island of thecomatosis.

DISCUSSION

Various authors [25–30] have described the enzyme pattern of the luteofollicular apparatus. Deane *et al.* [26] and Woodruff *et al.* [27] found lipids in the theca cells of growing and atretic follicles, while lipids are particularly abundant in the luteinized theca cells of the corpus luteum.

Like Foraker and Crespo [31] we could not demonstrate glycogen in the luteofollicular apparatus. Besides the theca cells, the granulosa cells of the cumulus oöphorus also possess some alkaline phosphatase activity [30]. In contrast to our findings and those of most authors, Deane *et al.* [26] did not report acid phosphatase activity in theca cells of growing follicles. König [30] found strong activity of this enzyme in the granulosa cells of Graafian follicles. Vessels are strongly coloured with the ATP-ase and the 5-nucleotidase technique. There is practically no enzyme activity in the luteofollicular apparatus. The strong 5-nucleotidase activity in the ovarian stroma is remarkable. Especially with the substrate α-naphthylacetate we found strong esterase activity in the theca cells of growing and atretic follicles. After rupture of the follicle the activity in the granulosa cells was very strong. McKay *et al.* [25] observed, in contrast to our results, a stronger esterase activity in the theca cells. Generally the activity of the enzyme system NADPH-tetrazolium reductase was stronger than the NADH-tetrazolium reductase. The theca cells showed stronger enzyme activity than the granulosa cells. The various dehydrogenases showed the same distribution as the corresponding tetrazolium reductase. In contrast to the other dehydrogenases the enzyme activity of α-glycerophosphate dehydrogenase was strongest in the granulosa cells. Different methods and substrates were used to demonstrate the 3β-ol hydroxysteroid dehydrogenase. The strongest activity was found with the substrate androstenediol by the method of Allen [12] (see Figs. 7 and 8). In the theca cells of both growing and atretic follicles, moderate light enzyme activity was demonstrated. The granulosa cells of the corpus luteum showed very strong enzyme activity. Ikonen *et al.* [32] also observed slight activity in the granulosa cells of atretic follicles. König [30] using different substrates demonstrated strong activity in the theca cells of atretic follicles derived from Graafian follicles.

We found strong secondary alcohol dehydrogenase activity in all theca cells and moderate activity in the granulosa cells of the corpus luteum. In contrast to the distribution of secondary alcohol dehydrogenase, the activity of alcohol dehydrogenase was stronger in the granulosa cells. The most important findings related to steroidogenesis are summarized in Table 3. Lipids, glucose-6-phosphate dehydro-

Table 3. Enzyme pattern of ovarian follicles, corpus luteum of menstruation, thecomatosis, thecoma and fibroma of the human ovary

	Growing follicles		Atretic follicles		Corpus luteum of menstruation		Thecomatosis		Thecoma		Fibroma
	T	G	T	G	T	G	L.C.	H.S.	L.C.	T.S	
Oil Red O	+/±	−	+	−	+++/++	++/+	+/±	±/−	+/±	++/+	+/−
Second. alcohol dehydr.	+++	−	+++/+	−	+++	+	+++	−	+++	−	−
Glucose-6-phosphate dehydr.	+++	+	+++	±/−	+++	++/+	+++	+	+++	+/±	±/−
3β-ol Hydroxysteroid dehydr.	+	−	±/−	−	±	+++	+	−	++	−	−
Non-specific esterases	+	−	+	−	+/−	+++	±	−	±	−	−

T=theca cells; G=granulosa cells; L.C.=luteinized cells; H.S.=hyperplastic ovarian cortical stroma; T.S.=thecal stroma.
−, ±, +, ++, +++ mean respectively: negative, slight, moderate, strong and very strong enzyme activity; /=varying activity.

genase and secondary alcohol dehydrogenase show the strongest activity in the theca cells of the corpus luteum. Glucose-6-phosphate dehydrogenase catalyses the initial oxidative step in the hexose-phosphate pathway of carbohydrate metabolism and is the generator of NADPH which is necessary for hydroxylation of the steroid molecule and for splitting the cholesterol side chain. Secondary alcohol dehydrogenase also seems to play a part in the cholesterol side chain cleavage [13]. It seems reasonable to postulate that the combination of Oil Red O positive material, glucose-6-phosphate dehydrogenase and secondary alcohol dehydrogenase reflects the beginning of steroidogenesis i.e. the production of pregnenolon. The enzyme histochemical findings indicate that this process is located in the theca cells of the growing and atretic follicles and of the corpus luteum. The enzyme 3β-ol hydroxysteroid dehydrogenase promotes the dehydrogenation of the various 3β-ol hydroxysteroids, the next step in steroidogenesis. Our findings suggest that this process is preferentially located in the theca cells of the growing and atretic follicles and particularly in the granulosa cells of the corpus luteum. The corresponding enzyme distribution of the non-specific esterases and of 3β-ol hydroxysteroid dehydrogenase is remarkable, but unexplained. Since cholesterol is present mainly as the ester of the 3β-hydroxy group of the molecule it could be possible that the 3β-hydroxysteroids are also present in the esterified form. In this case the saponification of the ester bond should precede the oxidation of the 3β-ol group.

During the preparation of this manuscript an article by Fienberg and Cohen [29] appeared. They described some enzyme activities in the stromal theca cells. The spindle stromal cell ("fibroblast") and the spindle stromal theca cell appeared identical in sections stained with haematoxylin and eosin. In groups of stromal theca cells, polygonal as well as spindle forms were found, together with transitional forms. These cells all showed a strong glucose-6-phosphate dehydrogenase activity. Only two postmenopausal patients' ovaries showed stromal theca cells with 3β-ol-hydroxysteroid dehydrogenase. Both patients had a proliferating endometrium although in one case there was also adenocarcinoma. The Sudan IV stain, by staining the lipid droplets, made some of the spindle stromal theca cells visible. These results are in agreement with our findings. Our patient (no. 6) showed the lipid band in the cortex described by these authors. In analysing menopausal ovaries associated with endometrial abnormalities, Novak *et al.* [33] found enzyme activity suggestive of steroidogenesis in two thirds of the cases. Despite the presence of an atrophic endometrium they found enzymatically active cells in the ovarian stroma in six of eighteen cases.

Savard *et al.* [34] stated that it is difficult to ascertain the importance of the stroma as a source of tangible quantities of steroid in the normally functioning ovary. Our experience with the enzyme histochemical methods described above is in agreement with this suggestion. It is unlikely that stromal cells of the normal ovary in adult life produce steroids.

In the senile ovary fibrous tissue penetrates the cortex and in advanced old age the cortex is often indistinguishable from old fibrous tissue elsewhere in the body [4]. Under certain pathological circumstances, probably by derangements of the hypothalamo-hypophysial system, proliferation of the multipotential stromal cells takes place and a proportion of these cells differentiate into steroid-producing cells.

Thecomatosis probably develops in this way and it may be a symptom of the syndrome of Stewart-Morgagni-Morel [35]. Some signs of this syndrome e.g. adiposity, diabetes mellitus and hypertension have also been recorded in patients with carcinoma of the endometrium [3]. A relationship between these symptoms is probable. A similar derangement may be responsible for hyperplasia of the hilus cells of the ovary [33]. One argument in support of this is that the combination of thecomatosis and hilus cell hyperplasia is not uncommon [3].

Charles *et al.* [7] could not demonstrate a relationship between hilus cell hyperplasia and cortical stromal hyperplasia on the one hand and urinary oestrogen output on the other. In our material we did not found islands of hyperplastic stroma without enzymatically active theca cells.

We suggest therefore that cortical stromal hyperplasia is simply one of the various grades of thecomatosis and we define the latter as hyperplasia of the cortical or medullary stroma with the formation of steroid-producing cells, whether the process is complete or not. Clearly enzymatic alterations will precede the development of the morphological characteristics of the theca cells.

Preliminary biochemical studies by Forleo [8] suggest that there is a considerable difference between steroid biosynthesis in pre- and postmenopausal women. He could not find any intermediate neutral steroids involved in the pathways from progesterone to oestrogens. However, the stroma cells of ovaries from postmenopausal women seemed able to perform

aromatization of testosterone into oestradiol.

The first observation is at variance with our findings as we showed that the stromal theca cells possess the enzymes required for the production of pregnenolone and to dehydrogenate the 3β-ol hydroxysteroids. In this way they may produce androgens and possible oestrogens. The second of Forleo's observations showed that this can in fact occur.

It is probably that oestrogens produced by the thecal stromal cells may play a part in the induction of some carcinomas of the endometrium. If thecomatosis is combined with a thecoma the likelihood of developing carcinoma increases. Our patient no. 15, who showed an increased oestrogen output in the urine, is a good example of the cases described in the literature [6].

Hilus cells are derived from mesenchymal cells that cannot be differentiated from fibroblasts [36]. Sometimes they are located around blood vessels or nerve fibres (Fig. 9). These cells which are consistently found in association with thecomatosis [3], showed the same enzyme pattern as the theca cells of the growing and atretic follicles, apart from the absence of the enzyme aminopeptidase. These findings are in agreement with those of Novak *et al.* [33]. They have the same enzyme pattern as the Leydig cells of the human testis [37]. Possibly the absence or presence of the enzyme aminopeptidase may be an indicator of the type of steroids that are produced. More work will have to be done to solve this problem. It is a well-known fact that hilus cell tumours may cause masculinization, so that they are able to produce androgens. In contrast to König [30], we, like Novak *et al.* [33] found 3β-ol-hydroxysteroid dehydrogenase in the hilus cells. It is yet impossible by enzyme histochemical methods to differentiate between androgen- and oestrogen production.

In sections stained with haematoxylin and eosin there was very little difference between the fibroblast of the ovarian stroma and the fibroma of patient 15.

Fibroma cells, in contrast to the normal stroma cell may possess some lipids and some acid phosphatase activity. Generally the enzyme pattern of the tumour resembled that of the hyperplastic stromal cells. The principal difference between the fibroma and the thecoma was the absence of cells with the enzyme pattern of theca cells. The most important enzymes for the differentiation being glucose-6-phosphate dehydrogenase, secondary alcohol dehydrogenase and the 3β-ol hydroxysteroid dehydrogenase (Table 2). The relationship between the ovarian abnormalities described are summarized in Table 4. The multipotential stromal cell is the source of many histopathological ovarian structures.

Further use of the enzyme histochemical methods described will give a better understanding of the ovarian abnormalities which may be partly responsible for the development of adenocarcinoma of the endometrium and for a more precise differentiation of ovarian tumours.

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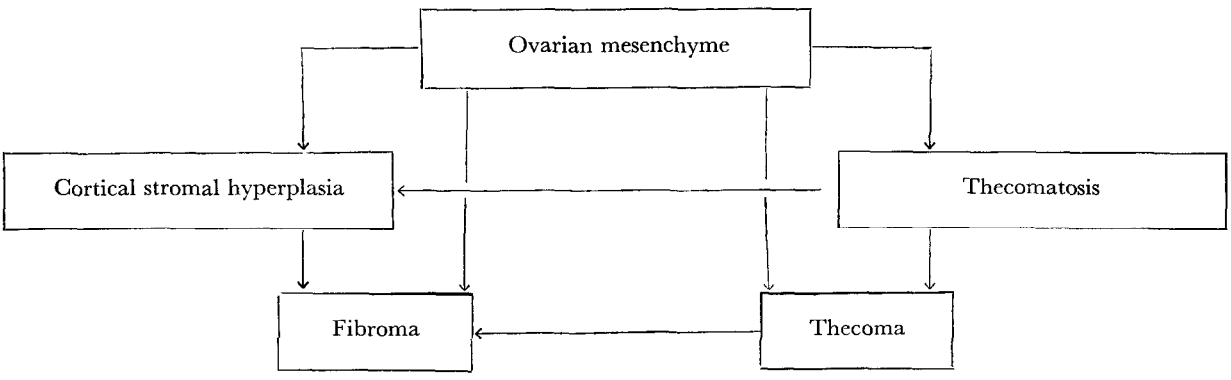
Table 4. The relationship between some human ovarian pathological conditions. The principle difference between cortical stromal hyperplasia and fibroma on the one hand and thecomatosis and thecoma on the other is the absence of steroid-producing cells



RÉSUMÉ

On a étudié la localisation de la production des stéroïdes ovariens par des méthodes histo-enzymologiques et les relations entre la cellule du stroma ovarien, la thécomatose, le fibrome et le thécome. Les cellules qui produisent des stéroides sont caractérisées par la déshydrogénase d'alcool secondaire et par la stéroïdo-3β-ol-déshydrogénase. Des cellules caractérisées par la présence de lipides, la phosphatase acide et la glucose-6-phosphodéshydrogénase, sont trouvées dans les îlots du stroma ovarien hyperplasique. Seule, la glucose-6-phosphate-déshydrogénase joue un rôle dans la production des stéroïdes. Il n'y a probablement pas de cas purs d'hyperplasie du stroma ovarien, mais seulement des stades différents de thécomatose. L'absence, ou la présence, de la stéroïdo-3β-ol-déshydrogénase et de la déshydrogénase d'alcool secondaire permet de différencier un thécome d'un fibrome. L'emploi ultérieur des méthodes histoenzymologiques peut aider à mieux comprendre les troubles ovariens consécutifs au développement du carcinome de l'endomètre et les aspects hormonaux des tumeurs de l'ovaire.

SUMMARY

The enzyme pattern of steroid-producing cells of the normal ovary had been investigated and discussed. The relationship between the ovarian stroma cell, thecomatosis, fibroma and thecoma of the ovary had been investigated. Steroid-producing cells are characterized by the enzymes secondary alcohol-dehydrogenase and 3β-ol hydroxysteroiddehydrogenase. Cells, characterized by the presence of lipids, acid phosphatase and glucose-6-phosphate dehydrogenase had been found in islands of hyperplastic ovarian stroma. Only the last-mentioned enzyme can play a part in steroidogenesis. Probably there are no pure cases of cortical stroma hyperplasia but only different grades of thecomatosis. The absence or presence of the enzyme 3β-ol hydroxysteroiddehydrogenase and secondary alcoholdehydrogenase are deciduous to differentiate a thecoma from a fibroma. Further use of the described enzymes can be helpful to get more insight in the ovarian abnormalities related to the development of the carcinoma of the endometrium and the hormonal aspects of ovarian tumours.

ZUSAMMENFASSUNG

Die Lokalisierung der Steroidsynthese in normalen Eierstocken wurde durch enzymhistochemische Methoden studiert. Die Beziehungen zwischen den Stromazellen des Eierstockes, der Thekomatose, dem Fibrom und dem Thekom wurden untersucht. Die Steroidsynthese in Zellen wurde durch die Enzyme sekundärer Alkoholdeshydrogenase und 3β-ol-Hydroxysteroiddeshydrogenase charakterisiert. In Inseln des hyperplastischen Stromas des Eierstockes wurden Zellen gefunden, die durch die Anwesenheit von Lipiden, sauerer Phosphatase und Glukose-6-Phosphat-Deshydrogenase charakterisiert sind. Nur das Enzym Glukose-6-Phosphat-Deshydrogenase kann in der Steroidsynthese eine Rolle spielen. Wahrscheinlich gibt es keine Fälle, in denen nur kortikale Hyperplasie des Stromas anwesend ist, sondern nur verschiedene Gradierungen von Thekomatose. Entscheidend in der Differenzierung zwischen Thekoma und Fibrom ist die Anwesenheit oder die Abwesenheit der Enzyme sekundärer Alkoholdeshydrogenase und 3β-ol-Hydroxysteroid Deshydrogenase. Eine weitere Verwendung der Enzyme kann zu einem besseren Verständnis der Eierstockstörungen leiten, die mit der Entwicklung des Endometriumkarzinoms und den hormonalen Aspekten der Eierstocktumoren verbunden sind.

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A Human Serum Component Required by the Cancer Coagulative Factor

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INTRODUCTION

WATER-SOLUBLE fractions with thromboplastic properties have been isolated from human cancer tissue and human chorion tissue by chromatography on DEAE-cellulose [1, 2] and by gel-filtration using Sephadex G.200 [3]. The location of the thromboplastic substance within the chorion cell has been investigated by Clarke [4] who reported high coagulative activity to be associated with the microsomal fraction. Thromboplastic activity associated with microsomal and other cell particles was mentioned by Seegers [5a] at an earlier date and, more recently, by Williams [6]. All the above-mentioned coagulative materials appear to have one or more characteristics in common with the lipoproteins and very high molecular-weight cell constituents isolated from bovine and human tissue by Chargaff [7–9].

The presence of thromboplastic activity in the cancer and chorion fractions was demonstrated either by the one-stage clotting test of Biggs and Macfarlane [10] using undiluted citrated human plasma, or by induction of clotting in the buffered diluted human plasma described by Boggust *et al.* [2]. The latter test system which has certain advantages, previously reported, was found to give results with tissue thromboplastins comparable with those obtained by the use of whole plasma in the conventional one-stage test [3].

The present paper describes the discovery in normal human serum of a component fraction with which the human chorion coagulative factor appears to react. For this reactant the term serum component is consequently used throughout. This reaction occurs prior to the formation of free thrombin and appears to be independent of the subsequent participation of prothrombin in the sequence. The reaction mixture differs from its components in its acquired property of converting prothrombin to thrombin.

This component has been isolated from human serum and is protein in nature. A fraction with corresponding activity has been prepared from human plasma and is referred to as plasma component, but as it is difficult to obtain the plasma component free from contaminating traces of prothrombin, which interfere with the investigation of the reaction mechanism, the present report is almost exclusively restricted to the serum component.

EXPERIMENTAL

The plasma component was prepared from fresh citrated pooled human plasma by a procedure identical with that described below for the serum component. Chromatography gave a single peak in the 1·0 molar phase and only this phase reacted characteristically with tissue extract. The pooled dialysed fractions of the 1·0 molar peak were tested, before and after heating for 30 min at 60°C, using chorion microsomes as tissue extract and bovine serum [5c] as an alternative source of activator. The plasma component, chorion microsomes and calcium chloride, with or without added activator were incubated for 15 min at 37°C. without the addition of a separate prothrombin fraction. Fibrinogen was then added, incub-

ation was continued and the times required for clot-formation were observed (Table 1).

The serum component was prepared from an eluted aluminium hydroxide adsorbate of serum by chromatography on DEAE cellulose. The part played by the serum component was investigated, using two-stage clotting tests

Preparation of Prothrombin

The preliminary adsorption step employed was similar to that described by Biggs and Macfarlane [10]. Fresh citrated blood-bank plasma (4·0 l.) was centrifuged for 20 min (9000 rev/min at 5°C) to remove fat and platelets.

Table 1

Plasma component	0·5 ml	Plasma component (heated)	0·5 ml
Microsomes (1·75 mg/ml)	0·5 ml	Microsomes (1·75 mg/ml)	0·5 ml
$CaCl_2 . 6H_2O$ (1·25%)	0·26 ml	$CaCl_2 . 6H_2O$ (1·25%)	0·26 ml
Fibrinogen (10 mg/ml)	0·2 ml	Fibrinogen (10 mg/ml)	0·2 ml
Time (min)	2·5	Time (min)	45
Plasma component (heated)	0·5 ml	Saline (no fraction)	0·5 ml
Microsomes (3·5 mg/ml)	0·25 ml	Microsomes (3·5 mg/ml)	0·25 ml
Bovine serum (1/100 in saline)	0·25 ml	Bovine serum (1/100 in saline)	0·25 ml
$CaCl_2 . 6H_2O$ (1·25%)	0·26 ml	$CaCl_2 . 6H_2O$ (1·25%)	0·26 ml
Fibrinogen (10 mg/ml)	0·2 ml	Fibrinogen (10 mg/ml)	0·2 ml
Time (min)	11·5	Time (min)	>50

which were set up in two independent series. In series A, the reaction between the serum component and tissue extract was assayed by timing clot-formation in recalcified citrated whole human plasma. The tissue extract employed in this case was the high molecular-weight fraction isolated from a supernatant of centrifuged homogenate of human chorion by gel-filtration in saline on Sephadex G.200. For the purposes of Series B, the reaction system was simplified in respect to the number of components by substituting for plasma a mixture of calcium with prothrombin and fibrinogen, the two latter being of human origin. Three different tissue extracts were used. These had been assayed for coagulative activity by the buffered diluted whole plasma method [2] and were diluted appropriately in saline to form preparations containing similar amounts of activity per unit volume when assayed by the plasma buffer method. The preparations used were: (a) a supernatant of homogenised chorion centrifuged for 20 min at 3500 rev/min to remove gross particulate matter; (b) the high molecular-weight fraction obtained from a) by gel-filtration; and (c) a suspension of chorion-cell microsomes.

Aluminium hydroxide gel was prepared by homogenising moist gel (B.D.H., Ltd. 5 g) with water (20 ml) to obtain a suspension. The suspension was added to the centrifuged plasma (6·5 ml for each 100 ml of plasma) and was stirred at 37°C for 30 min. The gel, after centrifuging for 20 min (9000 rev/min at 5°C), was washed by suspending in cold distilled water (100 ml for each 1000 ml of plasma) and centrifuging. The wash was repeated three times.

Adsorbed protein was eluted by stirring a suspension of the washed gel in phosphate buffer (0·066 M, pH 8·0: 80 ml of buffer for each 4000 ml of original centrifuged plasma) at 37°C for 1 hr, followed by centrifugation. After four successive elutions of the same gel, the combined solutions (320 ml containing 3·4 g of protein) were prepared for chromatography by dialysis against three changes, each of eighteen volumes, of ammonium acetate solution (0·1 M, pH 8·0).

A glass chromatography column (400×20 mm), maintained at 5°C by means of a cooling jacket, was prepared, containing DEAE-cellulose powder (40 g approx.) which had been equilibrated in ammonium acetate solution

(0·1 M, pH 8·0) and degassed *in vacuo* after washing by extraction successively in sodium hydroxide solution (0·5 N) and distilled water.

The prepared protein solution after dilution with an equal volume of 0·1 M ammonium acetate solution was applied to the column at a rate of approximately 1·5 ml/min and was eluted at a similar rate with 0·1, 0·2 and 1·0 M ammonium acetate at pH 8·0, and finally with sodium hydroxide solution (0·5N). The effluent was scanned continuously in an LKB Uvicord recording transmittance at $\lambda=253$ mμ and was collected automatically in fractions of 5 ml (Fig. 1). Elution with sodium hydroxide

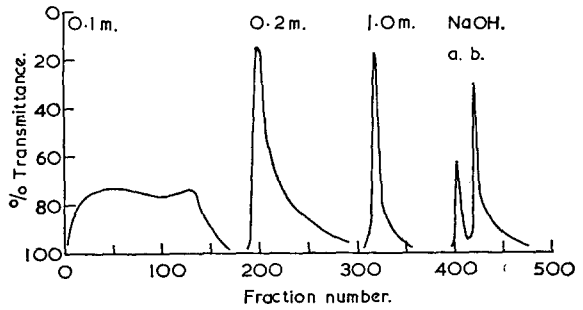

Fig. 1. *Chromatographic fractionation on DEAE cellulose of eluate from the aluminium hydroxide adsorbate of human plasma for isolation of prothrombin.*

yielded two phases which are referred to as NaOH*a* and NaOH*b*. Fractions corresponding to the principal protein phases were combined and dialysed against three changes, each of 18 vol. of 0·85% sodium chloride. Protein was estimated by the method of Folin and Ciocalteu, using human serum albumin (Mann Research Laboratories Inc., New York) as standard (Table 2). The pooled peaks were then assayed for prothrombin.

with calcium chloride solution (0·1 ml). The thromboplastin was prepared according to the manufacturer's directions and then further diluted before use (1 vol.+2 vol. of saline). The calcium chloride solution ($CaCl_2$. $6H_2O$, 0·6%) was prepared in saline containing bovine serum [5c] (1 vol. of serum + 99 vol. of saline). After 10 min incubation, a solution of human fibrinogen (0·1 ml: Kabi, Sweden; 5 mg/ml in a mixture of citrate–saline, 9 vol. and imidazole buffer, pH 7·3, 1 vol.) was added. The mixtures were again incubated at 37°C, examined at intervals and clotting times were recorded (Table 2). Portions of a solution of Parke-Davis Thrombin Topical in 30% glycerol containing 94 N.I.H. units per ml were diluted in saline to contain 9·4 and 0·94 units per ml. Portions of each thrombin dilution (0·3 ml) were added to saline (0·2 ml). The mixtures were incubated at 37°C for 10 min. Fibrinogen solution (0·1 ml, 5 mg/ml in saline–imidazole buffer) was added to each, the mixtures were further incubated at 37°C and examined at regular intervals. Assays of the dialysed fractions were repeated once; those of standard thrombin were repeated twice. Clotting times are recorded in Table 3. The NaOH*a* . phase (in saline) was heated for 45 min at 60°C to inactivate co-factors and then dispensed in 1·0 ml portions and stored at −25°C. Before use in the location of serum component in the serum chromatographic fractions, the prepared prothrombin, when assayed by the two-stage method according to Seegers [5b], was found to contain 40 units per ml. For use in the studies of serum component reaction mechanism, a portion was subjected to four-fold concentration by dialysis against polyethylene glycol at 4°C. This product, upon

Table 2

Phase	Eluent	Clotting times (sec)	Volume (ml)	Total protein (mg)
1	0·1 M amm. ac.	45, 65	650	390
2	0·2 M	350, 350	560	1550
3	1·0 M	∞ ∞	220	255
4a	0·5 N NaOH	15, 16	56	34
4b	0·5 N NaOH	∞ ∞	125	63

A preliminary location of prothrombin across the chromatographic fractions was carried out by the two-stage method adapted from Seegers [5d], using Parke-Davis Thrombin Topical as standard. A portion of the dialysed chromatographic fraction (0·3 ml) was incubated at 37°C with Difco rabbit brain thromboplastin (0·1 ml) in saline, together with calcium chloride solution (0·1 ml). The thromboplastin was prepared according to the manufacturer's directions and then further diluted before use (1 vol.+2 vol. of saline). The calcium chloride solution ($CaCl_2$. $6H_2O$, 0·6%) was prepared in saline containing bovine serum [5c] (1 vol. of serum + 99 vol. of saline). After 10 min incubation, a solution of human fibrinogen (0·1 ml: Kabi, Sweden; 5 mg/ml in a mixture of citrate–saline, 9 vol. and imidazole buffer, pH 7·3, 1 vol.) was added. The mixtures were again incubated at 37°C, examined at intervals and clotting times were recorded (Table 2). Portions of a solution of Parke-Davis Thrombin Topical in 30% glycerol containing 94 N.I.H. units per ml were diluted in saline to contain 9·4 and 0·94 units per ml. Portions of each thrombin dilution (0·3 ml) were added to saline (0·2 ml). The mixtures were incubated at 37°C for 10 min. Fibrinogen solution (0·1 ml, 5 mg/ml in saline–imidazole buffer) was added to each, the mixtures were further incubated at 37°C and examined at regular intervals. Assays of the dialysed fractions were repeated once; those of standard thrombin were repeated twice. Clotting times are recorded in Table 3. The NaOH*a* . phase (in saline) was heated for 45 min at 60°C to inactivate co-factors and then dispensed in 1·0 ml portions and stored at −25°C. Before use in the location of serum component in the serum chromatographic fractions, the prepared prothrombin, when assayed by the two-stage method according to Seegers [5b], was found to contain 40 units per ml. For use in the studies of serum component reaction mechanism, a portion was subjected to four-fold concentration by dialysis against polyethylene glycol at 4°C. This product, upon assay, contained 150 units per ml.

Preparation of serum component

Human serum (4·0 l.) was treated with aluminium hydroxide and the gel eluate was fractionated by column chromatography on DEAE-cellulose (Fig. 2) by methods identical with those described in this paper for the pre-

Table 3

Thrombin	Clotting Times
(units)	(sec)
0·94	75, 75, 64
9·4	15, 13, 16

paration of prothrombin from plasma. The principal fractions from each UV-absorbing phase were pooled, dialysed against three changes, each of 18 vol. of saline at 5°C, and assayed for protein content by the method of Folin and Ciocalteu, using human serum albumin as standard.

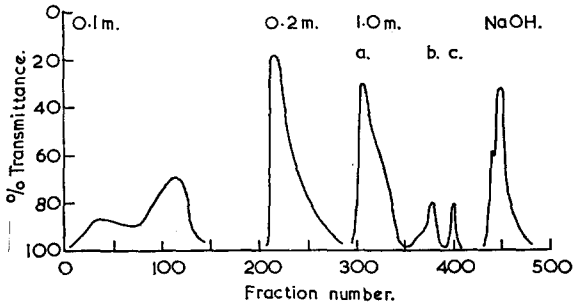

Fig. 2. *Chromatographic fractionation on DEAE cellulose of eluate from the aluminium hydroxide adsorbate of human serum for isolation of serum component.*

Location of activity in the chromatographic fractions was carried out in duplicate as follows. A saline suspension of microsomes (2 mg in 0·3 ml), the dialysed chromatographic serum fraction in saline (0·3 mg in 0·3 ml), calcium chloride solution ($CaCl_2 . 6H_2O$; 1·25% in saline, 0·26 ml) and prepared prothrombin (16 units in 0·4 ml) were mixed and incubated for 20 min at 37°C. A solution of human fibrinogen (Kabi, Sweden; 5 mg/ml in citrate-saline pH 6·5; 0·2 ml) was then added, the mixtures were incubated at 37°C and clotting times were recorded. Control assays, omitting the chromatographic materials, were set up simultaneously (Table 4). The active preparation was then dispensed in 1·0 ml portions (in saline solution) and stored at −25°C.

Preparation of chorion microsomes

Microsomes were prepared from human chorion by the method of Clarke [4] and then dialysed against water, freeze-dried, resuspended in saline (7 mg/ml) and stored at −25°C. (Found: N, 7·64%; P, 1·72%; inorganic residue, 3·16%; protein, 49·5%; chloroform-soluble lipoids, 37·5%. cf. Williams [6]: found for purified bovine lung microsomes: N, 7·6%; P, 1·6%; protein, 48·0%; phospholipid, 38·0%; cholesterol, 10·0%).

Serum component reaction mechanism

In Series B, for stage 1 of each test, the tissue extract (0·25 ml), the serum component solution (0·5 ml; 3·0 mg/ml) and calcium chloride solution (0·26 ml; $CaCl_2 . 6H_2O$ 1·25% in 0·1 M imidazole buffer pH 7·4) were preincubated together at 37°C for 20 min. For stage 2, solutions of prepared human prothrombin warmed to 37°C (0·25 ml; 150 units per ml) and of human fibrinogen (Kabi, Sweden; 0·2 ml; 10 mg/ml in saline–citrate, pH=6·5) were added prior to continued incubation and clotting times were recorded. Control experiments, omitting individual components, were set up simultaneously (Table 5).

In Series A, two-stage tests were set up in duplicate or triplicate and showed good agreement between individual results. In stage 1, the tissue extract (0·5 ml), serum component solution (0·5 ml, 3·0 mg/ml), and calcium chloride solution (0·26 ml, $CaCl_2 . 6H_2O$, 1·25%) were incubated together at 37°C for 20 min. In stage 2, a portion of the incubation mixture (0·2 ml) was then added to normal human citrated plasma (0·1 ml) prewarmed at 37°C. The mixtures were incubated at 37°C and the clotting times were recorded (Table 6). Control experiments were set up in which individual components were omitted or were heated at 60°C for 30 min before use in the test.

Table 4

Phase	Eluent ammonium acetate	Clotting times (min)		Volume (ml)	Total protein (mg)
1	0·1 M	26	23	475	570
2	0·2 M	17	19	300	750
3a	1·0 M	5	4	200	460
3b	1·0 M	11	13	80	30
3c	1·0 M	—	—	55	10
4	0·5 N NaOH	30	30	220	154
Control: No fraction		30		—	—

Table 5. (*Series B*)

(a)	Stage 1	G.200 Serum component Ca^{2+}	G.200 No serum component Ca^{2+}	No G.200 Serum component Ca^{2+}	G.200 Serum component Ca^{2+}
	Stage 2	Prothrombin Fibrinogen	Prothrombin Fibrinogen	Prothrombin Fibrinogen	No prothrombin Fibrinogen
	Time (min)	15	26	40	34
(b)	Stage 1	Microsomes Serum component Ca^{2+}	Microsomes No serum component Ca^{2+}	No microsomes Serum component Ca^{2+}	Microsomes Serum component Ca^{2+}
	Stage 2	Prothrombin Fibrinogen	Prothrombin Fibrinogen	Prothrombin Fibrinogen	No prothrombin Fibrinogen
	Time (min)	10	26	43	18
(c)	Stage 1	Chorion Serum component Ca^{2+}	Chorion No serum component Ca^{2+}	No chorion Serum component Ca^{2+}	Chorion Serum component Ca^{2+}
	Stage 2	Prothrombin Fibrinogen	Prothrombin Fibrinogen	Prothrombin Fibrinogen	No prothrombin Fibrinogen
	Time (min)	8	14	41	12

Table 6. (*Series A*)

Stage 1	Time (sec)	Stage 1	Time (sec)
1. T. extract Serum component Ca^{2+}	48, 50, 49	5. T. extract heated Serum component Ca^{2+}	77, 68, 70
2. T. extract No serum component Ca^{2+}	102, 94, 94	6. T. extract Serum component heated Ca^{2+}	85, 87, 87
3. No T. extract Serum component Ca^{2+}	110, 105, 109	7. Extract heated No serum component Ca^{2+}	175, 190
4. T. extract Serum component No Ca^{2+}	51, 58, 54	8. No T. extract Serum component heated Ca^{2+}	185, 196
		9. Control No stage 1	171, 181, 175

DISCUSSION

The component with which the cancer or chorion coagulative factor reacts can be separated from plasma in a group of proteins, including prothrombin, which are adsorbed by aluminium hydroxide gel as shown in Table 1. Serum gives a material with corresponding properties. On chromatographic fractionation of the material adsorbed from serum by gel, using DEAE cellulose, this component, separate from the prothrombin, was found in the fractions eluted by 1·0 molar ammonium acetate at pH 8·0. Of the three chromatographically distinct phases present in this group, two show definite activity; while the third, and smallest, has not been tested. For practical reasons, the first, most abundant and most reactive phase has been used as a source of serum component to investigate the cancer coagulative factor.

Human prothrombin used in the clotting test system to demonstrate the presence of the

serum component was prepared from plasma by a combination of gel adsorption, elution and chromatographic fractionation. The product thus obtained was heated to 60°C to render inactive labile accessory factors which are likely to include VII, IX and X, and possibly V [10]. Prothrombin values of the product thus obtained are relatively low in terms of activity (150 units per ml) on the basis of direct comparison with standard thrombin solution, the assay being carried out against human fibrinogen after conversion of the prothrombin to thrombin in the presence of thromboplastin, bovine serum accelerator globulin [5c], and ionised calcium. Nevertheless, sufficient potential activity is demonstrated in the prothrombin thus obtained to enable it to be employed as a useful test material for the assay of the thromboplastic activity of centrifuged chorion homogenate, the high molecular weight Sephadex G.200 fraction of centrifuged chorion homogenate and chorion microsomes.

The elimination, as far as possible, of accessory factors, other than those under immediate study from the test systems, and the exclusion of added phospholipids, is deliberate so as to enable a clearer picture to be obtained of the first stage in the reaction between a tissue extract and the component or components of plasma or serum which can give rise to a product capable of bringing about the conversion of prothrombin to thrombin. In consequence, in some of the test systems, clotting times have to be measured in minutes rather than the conventional seconds of the usual blood coagulation tests. In the context of fibrin thread formation by cancerous growths [11] and in tissue fluids, the time factor is not important since minutes, hours or even days may be required to establish the conditions necessary for clot formation to occur.

The tests in Series A (Table 6) show that tissue extract and the serum component promote clotting when added to a conventional plasma clotting system, either separately or together. Maximum reactivity is obtained when tissue extract and serum component are allowed to interact before addition to the plasma. Added calcium is not required for their interaction to take place. The serum component is thermolabile, and loses a great part of its reactivity after heating. In this it differs from prothrombin which is thermostable [10], and our prothrombin had been heated to inactivate accessory factors prior to its use in the tests.

These results are confirmed by the tests in Series B using tissue extract, human serum component, prepared human prothrombin and human fibrinogen solution. Maximum reactivity between serum component and tissue extract, whether in the form of chorion homogenate, microsomes or high molecular weight Sephadex G.200 fraction of chorion homogenate is found when they have been allowed to interact before addition to prothrombin and fibrinogen. Again the results indicate that the serum component does not contribute prothrombin to the test system, as is confirmed by the thermolability of the serum components. It is of interest to note that the tissue extracts together with serum component appear to possess the power of clotting fibrinogen in the absence of added prothrombin, whereas tissue extracts alone are without such coagulant activity [2]. While this is barely perceptible with the Sephadex G.200 fraction, it is well marked with chorion homogenate, microsomes being intermediate. While no complete explanation can be offered for this thrombin-like activity, the finding does not invalidate our observation that tissue extracts interact with a component found in serum and plasma to give a product further advanced in the thromboplastic sequence. It may be noted that Williams [6] found evidence for a similar type of reactivity with microsomes from bovine lung and a serum factor in the presence of added phospholipid. He suggested that the serum factor involved may be factor X.

No attempt has been made as yet to identify our serum component with any of the conventional clotting factors, since such identification demands clinical demonstration of a specific role in the correction of a clotting defect.

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RÉSUMÉ

On démontre que des éléments tissulaires labiles, obtenus de chorion et de cancer humains, réagissent de façon conjuguée avec un substrat provenant du sérum et du plasma humains pour donner un produit thromboplastique capable de faciliter la conversion de prothrombine en thrombine.

Le substrat diffère de la prothrombine par ses propriétés chromatographiques et sa thermolabilité, ainsi que par son comportement biologique.

On discute le rapport de cette découverte avec la formation de la fibrine dans la croissance des cancers humains.

SUMMARY

Evidence is adduced to show that labile tissue factors obtained from human chorion and cancer interact with a component found in human serum to give a thromboplastic product capable of promoting the conversion of prothrombin to thrombin.

The serum component differs from prothrombin in its chromatographic properties and in thermolability, as well as in its biological behaviour.

The relevance of this finding to the formation of fibrin by human cancers during their growth is discussed.

ZUSAMMENFASSUNG

Es wird nachgewiesen, dass aus Chorion und menschlichem Krebsgewebe gewonnene labile Gewebselemente mit einer gewissen Substanz des menschlichen Blutserums und des Plasmas verbunden reagieren, um ein thromboplastisch wirksames Produkt, das die Umwandlung des Prothrombins in Thrombin zu fördern imstande ist, abzugeben.

Dieser Wirkstoff unterscheidet sich von dem Prothrombin, sowohl was seine chromatographischen Eigenschaften betrifft, wie auch, was seine Thermolabilität und sein biologisches Verhalten anbelangt.

Das Verhältnis dieser Entdeckung zu der während des Wachstums des menschlichen Krebses beobachteten Bildung des Fibrins wird diskutiert.

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Possibilities for the Application of Fast Neutrons in Radiotherapy: Recovery and Oxygen Enhancement of Radiation Induced Damage in Relation to Linear Energy Transfer*

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INTRODUCTION

RADIOTHERAPY of malignant tumors has during the 60 years of its application depended almost entirely on the use of the most readily available ionizing radiations namely X-rays, γ-rays and fast electrons. A characteristic property of these radiations is their low mean linear energy transfer (LET) which ranges from about 0·2 to a few keV/μ of unit density tissue. Although various other existing types of radiation, which are characterized by energy dissipation properties different from X- and γ-rays, are of great interest for the investigation of basic mechanisms in radiobiology, they cannot in general be employed for the treatment of clinical tumors, because of insufficient penetration into tissue or the lack of intense beams. One notable exception is provided by fast neutrons, which can be produced with high yields by bombardment of targets of deuterium, tritium or beryllium with for instance protons or deuterons accelerated in cyclotrons or other accelerators.

X- and γ-rays dissipate energy mainly by interactions with electrons in the irradiated material, through Compton scattering and photoelectric absorption. Fast neutrons with energies between 0·1 and 10 MeV dissipate energy in tissue mainly by interactions with hydrogen nuclei and the resulting recoil protons have a mean linear energy transfer in excess of 10 keV/μ. At neutron energies in excess of 10 MeV, interactions with C-, N- and O-nuclei become important and part of these give rise to a component with LET values in excess of 100 keV/μ.

In order to indicate briefly the fundamental importance of differences in LET with regard to the energy dissipation by radiations in cells and subcellular structures, reference may be made to Fig. 1 where a schematic representation is given of the distribution of energy dissipated in small volumes in a cell, resulting from irradiation with γ-rays (A) and densely ionizing particles (B). The fact that the high local concentration of energy deposition along relatively few tracks, as produced by high LET radiations, can cause biological effects which are different from the more uniformly distributed energy dissipation produced by X- and

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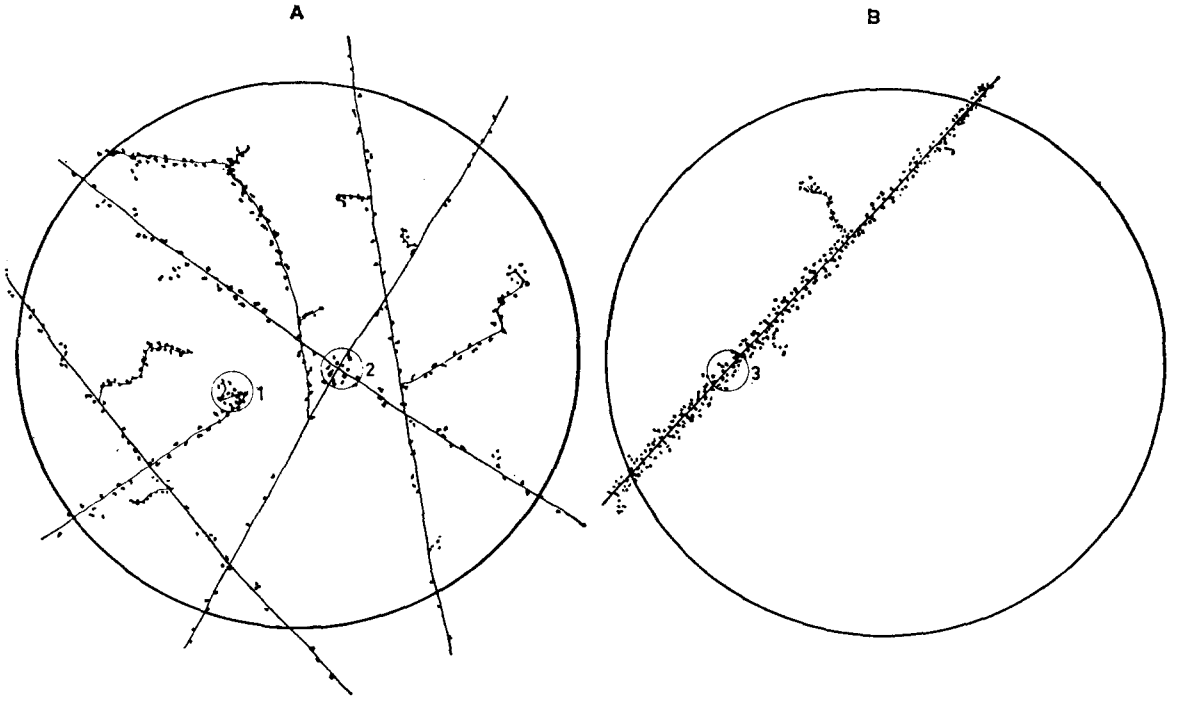

Fig. 1. Schematic representation of energy dissipation patterns in small volumes in cells irradiated with equal doses of γ-rays (A) or high LET particles (B). High concentrations of energy dissipation events occur in both (small circles 1, 2 and 3), but are per unit dose less frequent in A than in B.

γ-rays, has been known for a long time [1]. Quantitative as well as qualitative differences have been demonstrated with various systems and some of these will be described later on [2].

Interest in the effects of high LET radiations on tumors stems from the expectation that in comparison with X- and γ-rays an increased discrimination might be obtained between damage to cancer tissue and normal tissue respectively. If the aim of the radiotherapist can be described as the destruction of all tumor cells without causing irreparable damage to normal tissue, then it will be clear that the limitation of radiotherapeutic possibilities results from the dose received by that part of the normal tissue which is unavoidably included in the high dose treatment region. It is probably superfluous to state that the dose administered to the tumor frequently cannot be made large enough because of this limitation and that any possibility which may provide an improvement in the relative effectiveness for producing damage to tumor tissue as compared with normal tissue, sometimes called therapeutic ratio, must be investigated.

The first radiobiological experiments with cyclotron-produced fast neutrons were carried out by Lawrence, Aebersold and Lawrence and these resulted only a few years after their discovery in the first application of neutrons in the radiotherapy of 249 patients by Stone, Lawrence and Aebersold between 1937 and 1943 [3]. Taking into acccount the fact that almost all cases concerned advanced cancer, considered incurable by any conventional technique, the responses of the tumors were satisfactory in various cases. Nevertheless application of this treatment was discontinued after 1943 because serious late effects on skin and subcutaneous tissue were observed. It was concluded that for these late effects in normal tissue the relative biological effectiveness (RBE) of fast neutrons was higher than for early effects on skin, e.g. erythema and for the killing of tumor cells. This conclusion has effectively discouraged any further trials of the use of fast neutrons in radiotherapy ever since. It is important to note that in addition to the implied high RBE for late effects on normal tissue, the fast neutrons used, produced with the 37-in. and 60-in. cyclotrons at Berkeley, with modal energies of 4 and 7 MeV respectively, had some less favorable characteristics in other respects. Penetration into tissue is comparable with that of 200–250 kV X-rays and scattering is not at all negligible. Furthermore these early cyclotrons were not as dependable as would have been desirable for a regular course of radiotherapy and this resulted in variations in the dose fractions, the total doses and the over-all time of the treatments.

The aim of the discussion to be presented in the next sections is to investigate which factors might have contributed to the conclusion of an unduly high RBE of fast neutrons for adverse

late effects on normal tissue, and whether an understanding of these factors will provide an insight in the possible causes and indicate ways to avoid them. Subsequently the possible advantages of fast neutrons for radiotherapy will be discussed and finally the neutron energy most suitable for this application will be estimated.

The initiative to re-evaluate the possibilities of the use of fast neutrons in radiotherapy was taken by Prof. Fowler and collaborators, working with fast neutrons produced by the MRC cyclotron at Hammersmith Hospital in London [20]. These investigators measured the RBE of fast neutrons relative to X-rays for skin damage in pigs after exposure to single and fractionated doses. They found higher RBE values in fractionated regimes as compared with single exposures and suggested that this difference might have contributed to Stone's finding of severe skin and subcutaneous tissue damage [4–7]. A satisfactory interpretation of these findings can be derived from results of radiobiological experiments with densely and sparsely ionizing radiations, which show that survival curve shapes are different for these different radiations and that this corresponds with differences in recovery of part of the damage [8–10].

2. FUNDAMENTAL RADIOBIOLOGICAL DATA CONCERNING DAMAGE PRODUCED BY RADIATIONS OF DIFFERENT LET

The experimental data which are of interest for the present discussion have been obtained during the past several years mainly as a result of the development of techniques which made possible the quantitative determination of the fraction of cells in a population, which after irradiation have retained the capacity to produce an unlimited number of descendents. This fraction is usually referred to as surviving fraction. It is outside the scope of this discussion to describe these techniques in full, but briefly systems are employed in which cells are plated *in vitro* or inoculated in recipient animals in such a way that descendents of individual cells are allowed to proliferate till they can be scored, either as a clone in case Puck's *in vitro* cell culture technique is used or as a tumor in case Hewitt's tumor cell transplantation technique is employed [11, 12]. For purposes of demonstration I will mainly describe results obtained by ourselves with the tissue culture system, although in various cases similar results have been published by others using different systems.

The techniques mentioned enable the determination of survival curves, examples of which are presented in Fig. 2 for α-radiation from ^{210}Po with a LET of 140 keV/μ and 250 kVp X-rays with a mean LET of about 2 keV/μ [10]. Before discussing characteristic properties of these curves and the possibilities to modify them, it is worthwhile to make a remark on the relevance of these curves with regard to tumor therapy. It may readily be appreciated that the aim of a radiotherapeutic treatment with respect to the tumor, is attained if the capacity for unlimited proliferation of all tumor cells is destroyed. In terms of survival this implies, that the probability for the cure of a tumor containing 10^{10} tumor cells, treated with a dose which corresponds to a surviving fraction of 10^{-11}, is 90 per cent. In order to estimate the dose required for this effect we have to extrapolate the experimental curves to the surviving fraction of 10^{-11}, as shown in Fig. 2. For single doses of 250 kVp X-rays we arrive then at a dose of approximately 2650 rad and for 3·4 MeV α-radiation at a dose of about 1650 rad. From this example a relative biological effectiveness (RBE) of about 1·6 can be calculated for this α-radiation relative to X-rays, because the RBE is defined as the ratio of two doses required to produce quantitatively equal effects. With regard to damage produced by irradiation of organized tissues, it is less simple to show that survival curves are relevant. Evidence for this hypothesis can only be obtained by indirect ways. Studies of effects of fractionated doses on pig skin and on mortality of mice due to intestinal damage, have demonstrated for instance that the responses to fractionation closely resemble the responses of single cell populations tested *in vivo* and *in vitro* [13]. It is impossible, however, to show that systemic factors do not play a part at all in the expression of damage in organized tissue.

We will next discuss various characteristics of survival curves obtained for different radiations which cause the RBE not to represent a single value, but which make it dependent on a number of factors. These factors are:

(a) *Shapes of survival curves* — The survival curves for low LET radiations are in general different in shape from the curves obtained with high LET radiations. With high LET radiations exponential survival curves are obtained whereas with low LET radiations the curves generally exhibit an initial region of low slope followed by a steeper part at higher doses. This is exemplified in Fig. 2. As a consequence the RBE is dependent on the surviving fraction considered. At 10 per cent survival for instance

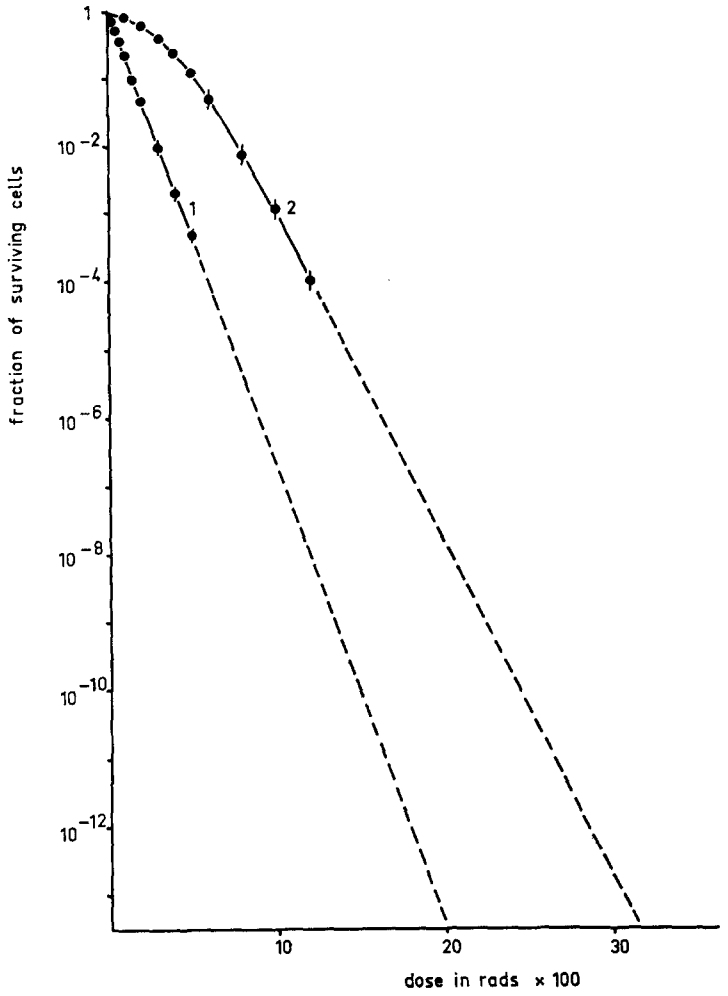

Fig. 2. Dose–survival curves of T-1 cells (cell line originally derived from human kidney), irradiated with single doses of 3·4 MeV α-radiation of high LET (curve 1) and with single doses of 250 kVp X-rays of low mean LET (curve 2).

the curves of Fig. 2 show that α-radiation has a RBE of about 3·5 relative to 250 kVp X-rays as compared with the factor of 1·6 calculated for a surviving fraction of 10^{-11}. At very low doses the RBE may attain values as high as 8.

(b) *Recovery of sublethal damage* — The shape of the survival curves obtained with 250 kVp X-rays shows that the slope increases with increasing dose in the range measured. It may be concluded that at least part of the cell killing produced by low LET radiation is due to accumulation of damage. This implies that sublethal damage is produced which may become lethal if enough is accumulated. Elkind has first demonstrated and this has been confirmed by many investigators including ourselves, that in individual cells recovery of sublethal damage occurs within a few hours after irradiation and after this time interval, surviving cells respond quantitatively to a subsequent irradiation as if they have not been irradiated at all [14]. As a consequence fractionated irradiation is less effective in killing cells than single doses, as exemplified in Fig. 3, curves 4 and 5. In experiments with cultured human cells we have shown that the survival curves obtained with high LET radiations show only a slight curvature or none at all and as a consequence little or no effect of fractionation is observed [2, 9]. It can be concluded therefore that with fractionated irradiation the RBE of high LET radiation is larger than with equivalent single doses. It will be clear that these RBE variations result primarily from the variations in the effectiveness of low LET radiations with fractionation.

(c) *The oxygen effect* — It has been observed with many systems that the presence of oxygen is an important factor with regard to the radiosensitivity of cells. The enhancing action of oxygen can be expressed quantitatively by a dose-modifying factor, generally called the oxygen enhancement ratio (OER). The OER is known to depend on the type of ionizing

radiation employed and decreases with increasing LET. This difference is exemplified in Fig. 3 where the OER of 3·4 MeV α-particles is shown to be equal to about 1·15 as compared with 2·7 for 250 kVp X-rays. In Fig. 4 similar data are presented for 15 MeV neutrons and fission spectrum fast neutrons with a modal energy value of 1·5 MeV. It is clear that because of these low OER values for high LET radiations, the RBE values of α-particles and fast neutrons are higher for cells in anoxic conditions as compared with the RBE of these radiations for cells in oxygenated conditions. In addition, it is shown in Fig. 3 that recovery of sublethal damage is also observed if cells are irradiated in anoxic conditions.

3. DISCUSSION OF THE SIGNIFICANCE OF RBE VARIATIONS FOR THE INTERPRETATION OF CLINICAL RESULTS OF FAST NEUTRON RADIOTHERAPY

The main conclusion which has been drawn by Stone, Lawrence and Aebersold from the clinical results of treatments of 249 patients, implies that the RBE value obtained for a threshold skin reaction (erythema) is not valid for the epidermolytic reaction and for the late atrophy and fibrosis reaction of the skin and subcutaneous tissue. The RBE of fast neutrons was found to be considerably higher for the latter effects.

If the "neutron unit" *n* which was used at the time when these data were obtained, is taken to

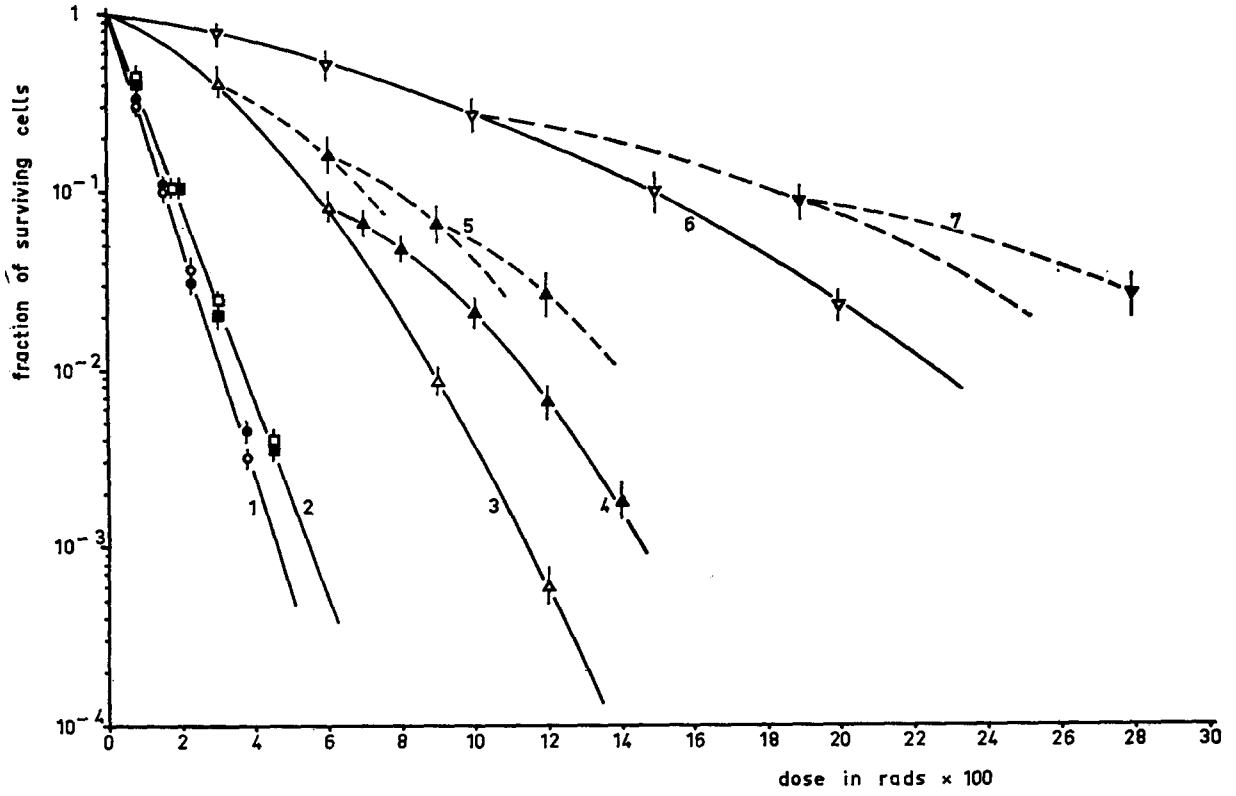

Fig. 3. Effects of dose fractionation and anoxic conditions on the survival of T-1$_g$ cells irradiated with α-particles from ^{210}Po and 250 kVp X-rays. Open symbols refer to single exposures, closed symbols refer to fractionated exposures.

Curve 1: Survival curve of cells in equilibrium with air. Open circles: single exposures to 3·4 MeV α-radiation; closed circles: doses of 3·4 MeV α-radiation fractionated in two equal parts administered with intervals of 8 hr.

Curve 2: Survival curve of cells equilibrated with pure nitrogen 10 min prior to and during irradiation. Open squares: single exposures to 3·4 MeV α-radiation; closed squares: doses of 3·4 MeV α-radiation fractionated in two equal parts administered with interval of 8 hr.

Curve 3: Survival curve of cells in equilibrium with air, single exposures to 250 kVp X-rays.

Curve 4: Survival curve of cells in equilibium with air irradiated with a first dose of 600 rad of 250 kVp X-rays, after an 8-hr interval, second doses of 100, 200 etc. rad of 250 kVp X-rays.

Curve 5: Survival of cells in equilibrium with air, exposed to repeated doses of 300 rad of 250 kVp X-rays, intervals of 3 hr

Curve 6: Survival curve of cells equilibrated with pure nitrogen 10 min prior to and during irradiation with doses of 250 kVp X-rays.

Curve 7: Survival of cells equilibrated with pure nitrogen exposed to repeated doses of 900 rad of 250 kVp X-rays, intervals of 3 hr.

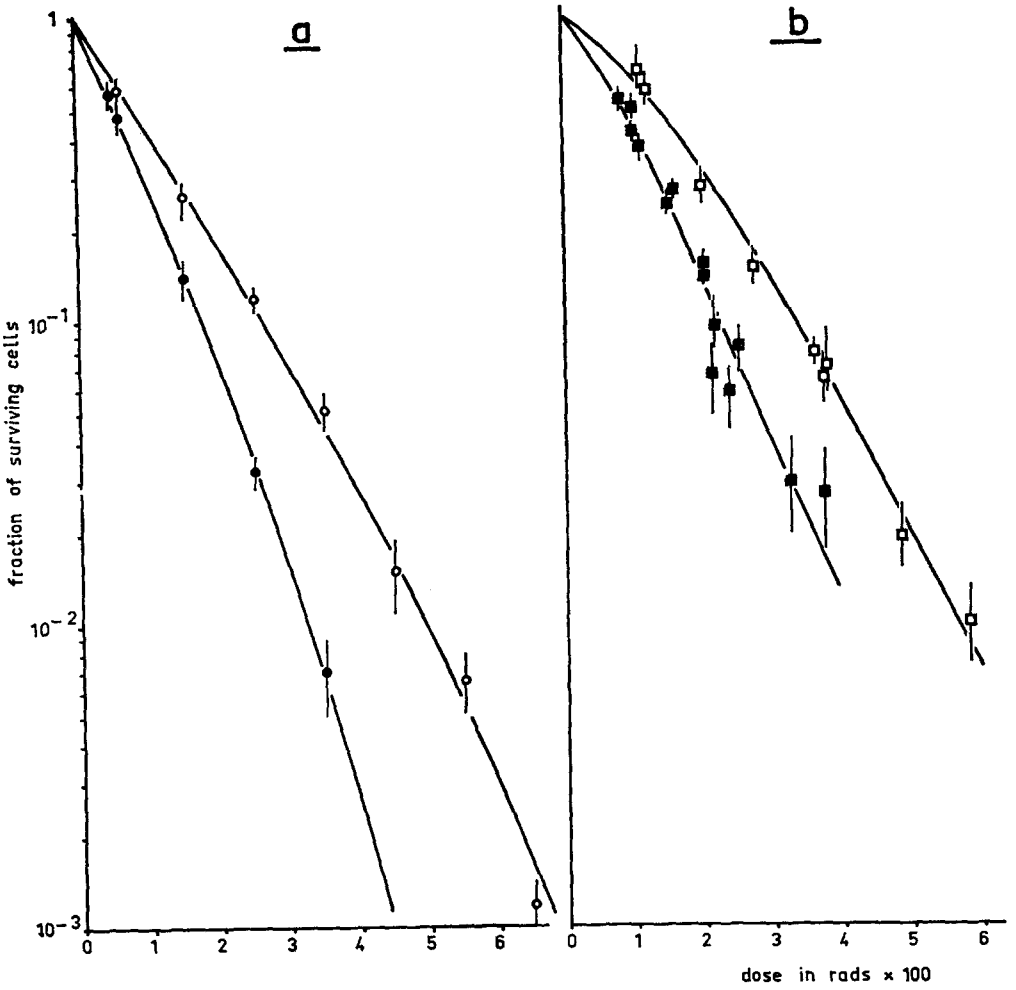

Fig. 4. Survival curves for cultured cells of human origin (T-cells) irradiated in oxygenated and anoxic conditions with fission spectrum fast neutrons (a) and with 15 MeV neutrons (b). Closed symbols refer to oxygenated cells and open symbols to anoxic cells.

be equal to 2 rad, an RBE of these neutrons for the threshold erythema may be estimated at 3–3·5. This value may be assumed to correspond to a dose of about 600 rad of X-rays relative to a dose of about 200 rad of fast neutrons. This RBE value of 3 could according to Stone not be applied to the adverse late effects for which a higher RBE was implied.

We may now inquire whether this effect can be understood on the basis of the radiobiological data discussed. The radiotherapeutic treatment of patients with fast neutrons at Berkeley was carried out with total doses ranging from about 800 to 2000 rad. The fractionation schemes varied almost from patient to patient, total doses were given in 3–19 fractions with total duration between 8 and 48 days and individual fractions varied between 50 and 400 rad [3, 15]. These differences may not have been very important, however, with respect to the effects of fast neutrons because, as shown in the previous section, fractionation influences the total amount of damage produced by high LET radiations to a much smaller extent than damage produced by X-rays and γ-rays. It may be noted that the clinical results described by Stone *et al.* indicate that regardless of the fractionation schemes, serious late damage to irradiated normal tissues was observed. This relatively small effect of the mode of fractionation is for survival of cultured cells after irradiation with fast neutrons, exemplified in Fig. 5, curves 2 and 3. In addition, results obtained by Fowler *et al.* concerning damage to the skin of pigs show also less recovery after fast neutron irradiation as compared with X-irradiation [20]. Both clinical and experimental data therefore are in agreement with the hypothesis that also with respect to damage to organized tissues, the effects of fractionation of doses of fast neutron radiation are relatively small.

With low LET radiation a quite different result is obtained. Large dose fractions of X- or

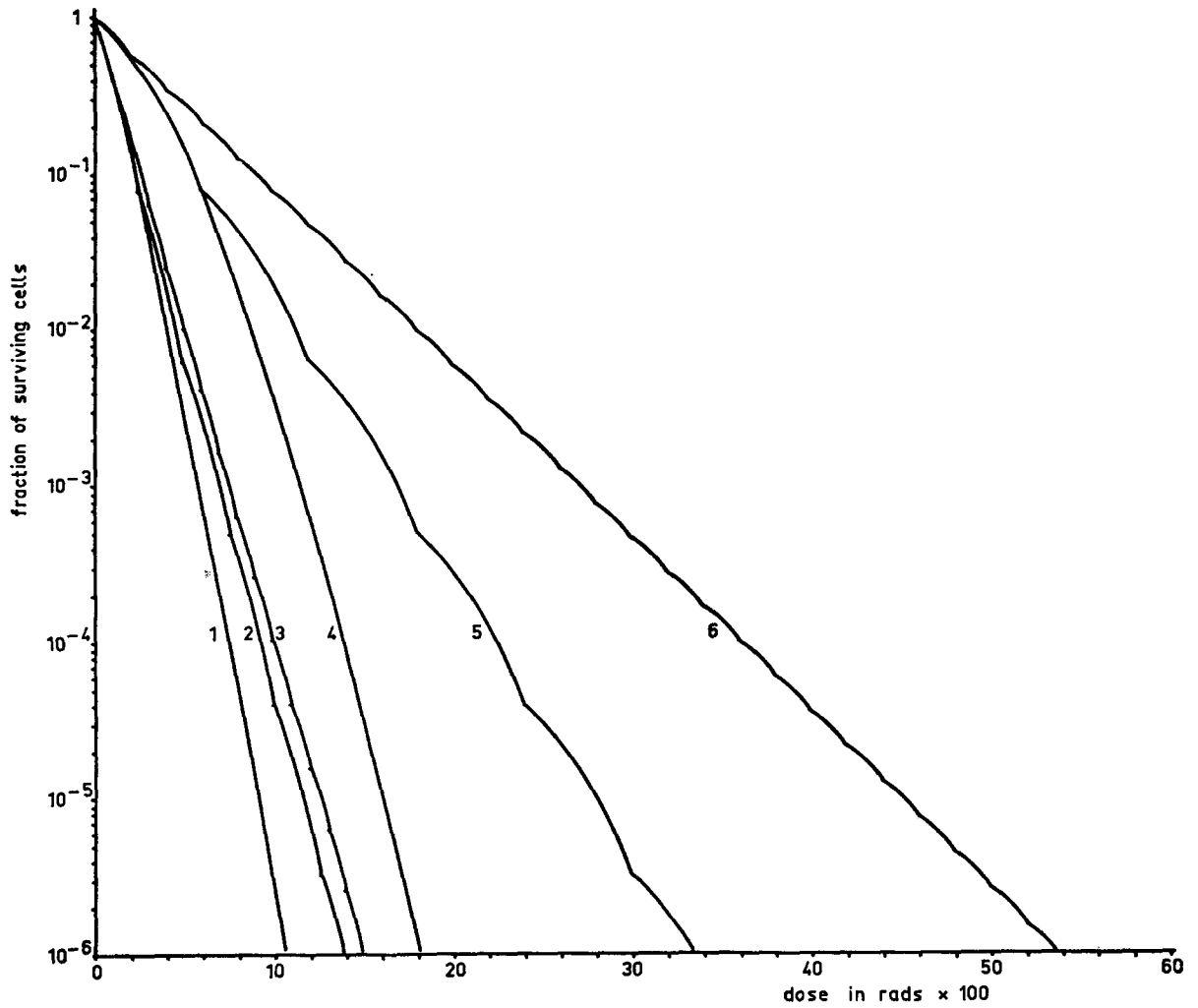

Fig. 5. Theoretical survival curves of cells irradiated with different dose fractions assuming complete repair of sublethal damage in the intervals. Curves 1 and 4 for single doses are based on experimental data obtained with cyclotron produced fast neutrons and 250 kVp X-rays respectively. Curves 2 and 3 demonstrate the effects of fractionation of fast neutrons. Curves 5 and 6 demonstrate the effect of fractionation of 250 kVp X-rays.

γ-rays, e.g. of 600 rad, are much more effective in reducing the fraction of surviving cells as compared with equal total doses administered in small fractions, e.g. of 200 rad, as exemplified by curves 5 and 6 of Fig. 5. For the production of a threshold skin erythema reaction, a dose of about 600 rad was estimated to be equivalent to 200 rad of fast neutrons. However, in radiotherapy it is usual to administer dose fractions of between 200 and 300 rad, i.e. at most half the dose required to produce the threshold erythema reaction. This difference in size of individual dose fractions of X- or γ-rays is very important with regard to the RBE of fast neutrons relative to X-rays. It may be derived from Fig. 5 that with dose fractions of 200 rad, X-rays are for equal total doses less effective by a factor of about 1·6 than with dose fractions of 600 rad. Therefore, if it is assumed that survival curve characteristics are relevant for the effects on organized tissues, the hypothesis may be advanced that the RBE for late effects is higher than for the threshold erythema produced by single doses, not because the effects of neutrons are so much dependent on the size of the individual dose fractions, but because for the standard of reference, i.e. low LET radiation, the size of the individual dose fractions is extremely important. Consequently, with regard to the effects on tumor cells as well as on normal tissues, the RBE of fast neutrons relative to X-rays administered in a treatment course in daily dose fractions of, e.g. 200 rad will be higher than the RBE of these neutrons relative to X-rays administered in a single dose of 600 rad. Thus the doses of fast neutrons administered by Stone *et al.* which, with an RBE value of 3·0 derived from the erythema reaction, were believed to be equivalent to 2700–7000 rad of X-rays, may be estimated to be comparable with effects of doses of X-rays which are a factor of about 1·5 higher, i.e. equivalent to about

4000–10 000 rad of X-rays. From these considerations it may be concluded that adverse late sequelae observed after the application of fast neutrons may have been due at least partly to over-expsoure and that by the use of lower doses these effects might have been avoided without increasing the probability of recurrence of the tumors considerably. It should be emphasized, however, that no certainty on this point exists and that in addition to the difference between low LET and high LET radiations with regard to the intracellular recovery, there may exist differences in other respects. Experiments carried out by Fowler concerning skin damage in pigs have not shown specific lesions or unduely high RBE values of fast neutrons for late effects however [5].

4. POSSIBLE ADVANTAGE OF FAST NEUTRON RADIOTHERAPY

Obviously it is not sufficient to suggest that fast neutrons do not have to be particularly harmful if proper doses, based on adequate knowledge of fundamental radiobiology, are employed. A renewed interest in this type of radiation with a view of its application in clinical radiotherapy is only justified if the existence of a definite advantage over low LET radiations, e.g. X- and γ-rays can be demonstrated. Such a possiblity of an advantage of high LET radiations stems from the observation discussed earlier that anoxic cells are less sensitive to X- and γ-rays by a factor of 2·5–3 as compared with oxygenated cells, whereas with high LET radiations the OER may be much smaller. Evidence is accumulating that many tumors, even those without grossly necrotic regions, contain anoxic cells as a result of insufficient blood supply to that area. For these anoxic cells the fraction of cells surviving a given dose will be significant, even after irradiation with doses which are sufficient to kill all well oxygenated cells. This is exemplified in Fig. 6. From an extrapolation of the experimental curves it can be estimated that at a single dose of 2650 rad of X-rays, the oxygenated cell population is reduced a fraction 10^{-11}, but the anoxic population only to a fraction 10^{-3}. An obvious solution to this problem would be to increase the oxygen supply but this is not simple to achieve although work along this line is being carried out, using high pressure oxygen tanks.

As discussed in a previous section, with high LET radiations the difference between the sensitivities of oxygenated and anoxic cells is smaller than with X- or γ-rays. Therefore with the application of these radiations the disadvantage of well oxygenated normal tissues as compared with the anoxic cells in a tumor would be smaller than with low LET radiations. This is exemplified in Fig. 6 for cell populations containing 100 per cent, 10 per cent and 1 per cent of anoxic cells. The maximum gain factor, defined as the ratio of OER values of X-rays and fast neutrons respectively, is obtained if all cells in the tumor are anoxic, and it decreases with decreasing fraction of anoxic cells and with the dose administered [6, 7]. This causes considerable uncertainty about the actual gain to be expected from the use of fast neutrons. Different tumors and tumors in different sites may contain various fractions of anoxic cells. Powers and Tolmach measured survival curves for tumor cells irradiated *in vivo* which were consistent with the assumption that 1 per cent of the cells in a solid transplantable lymphosarcoma in the mouse was anoxic [16]. Reinhold studied a rhabdomyosarcoma transplantable in a rat and van Putten and Kallman investigated C3H/Km sarcoma transplantable in mice. They obtained survival curves which indicate that 10–20 per cent of the cells in these tumors are anoxic [17, 18]. If fractions of anoxic cells in human tumors are assumed to be of the same order, then it can be deduced from Fig. 6 that at surviving fractions of 10^{-4} and less, fast neutrons would be considerably more effective than X- or γ-rays.

The advantage of the use of fast neutrons in killing anoxic cells in tumors pertains with certainty to tumors irradiated with single large doses of radiation. If fractionation is employed another variable is introduced. With small single doses the gain factor is smaller than with large doses (see Fig. 6) and consequently if a large total dose is administered in small fractions the gain factor of neutrons will depend on the size of the fractions, the fraction of anoxic cells and, in addition, on the response of the tumor to irradiation with respect to the fraction of anoxic cells present. If the well oxygenated population is reduced by a given factor as a result of a first dose of radiation, cells previously anoxic may become oxygenated because the killed cells may consume less oxygen or disintegrate. The situation may therefore be very complicated indeed and it may be different for different tumors. It will be clear, however, that fast neutrons will only give an advantage over low LET radiations if a high proportion of cells is anoxic and if relatively large dose fractions can be employed. Expressed in another way, it can be stated that fast neutrons can be expected to be of advantage for the treatment of those tumors which are highly radioresistant to X- and

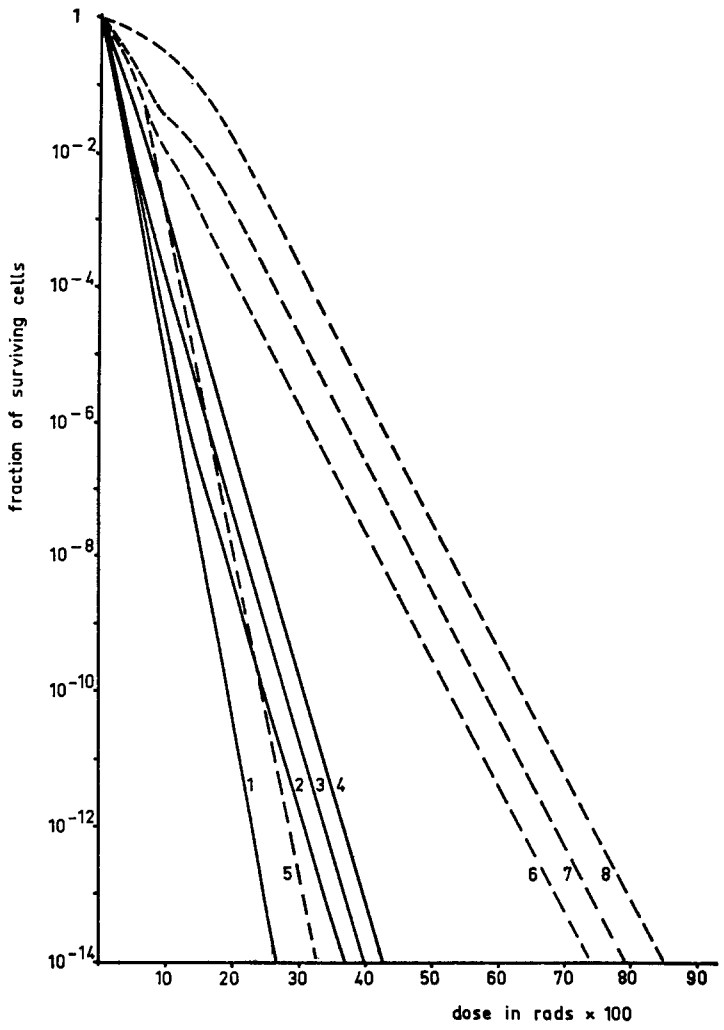

Fig. 6. *Theoretical survival curves of cells irradiated with single doses of* 15 *MeV neutrons* (*curves* 1 *to* 4) *and* 250 *kVp X-rays* (*curves* 5 *to* 8). *Curves* 1 *and* 5 *have been derived from experimental data obtained with oxygenated cells. Curves* 4 *and* 8 *have been derived from experimental data obtained with anoxic cells. Curves* 3 *and* 7 *have been calculated from curves* 1, 4, 5 *and* 8 *assuming* 10 *per cent of the cells in the irradiated population are anoxic. Curves* 2 *and* 6 *have been calculated from curves* 1, 4, 5, *and* 8 *assuming* 1 *per cent of the cells in the irradiated population are anoxic.*

γ-rays or electrons, presumably because they contain a high proportion of anoxic cells and because they show no response to irradiation with dose fractions of a few hundred rad. Little or no advantage would be expected for tumors which are clinically radiosensitive to X- and γ-rays presumably because these are well oxygenated and the use of dose fractionation improves the radiotherapeutic ratio.

Evidence that different types of cells may differ with respect to the effects of fractionation is accumulating and should be investigated more extensively.

6. DISCUSSION OF THE OPTIMAL ENERGY OF FAST NEUTRONS TO BE EMPLOYED FOR RADIOTHERAPY

Assessment of the possibilities concerning the application of fast neutrons in radiotherapy is clearly not only dependent on the oxygen enhancement ratio, but a number of other factors must be considered as well, notably depth dose characteristics, collimation and the dose distribution outside the treated area. In addition fractionation effects should be studied. It is outside the scope of this contribution to discuss all of these factors, but it is important to note that for some of these insufficient experimental data are yet available.

About two characteristics a few remarks will be made.

(a) *Neutron energies and depth doses characteristics* — Penetration of fast neutrons into tissue increases with neutron energy. Neutrons with energies of 5 MeV or less must be considered unsuitable except for superficial tumors. Fast neutrons produced by bombarding a Be target with deuterons of 15 MeV energy, as used by

Stone, Aebersold and Lawrence, are comparable with the fast neutrons produced by the Medical Research Council cyclotron at Hammersmith Hospital in London and exhibit a spectrum of energies ranging from 0–20 MeV, with maximum intensity at about 6 MeV [19]. This radiation is characterized by depth dose distribution equivalent to 250 kV X-rays and these would probably be considered to be the minimum acceptable in the present decade, in which multi-MeV X- and γ-rays become readily available. With increasing energy of the neutrons, depth dose characteristics improve. Mono-energetic 15 MeV neutrons, which can be produced by bombarding tritium targets with deuterons accelerated to 0·1 MeV or more, have very good penetration, comparable with multi-MeV X-rays. For the production of these neutrons with a sufficiently high output, an important problem is the construction of a suitable target. Present targets do not as yet have a half-life in excess of 10–15 min for adequate outputs. However, research in this field is actively being pursued.

(b) *Neutron energies and oxygen enhancement ratios* — OER values reported in the literature indicate an increase of the oxygen enhancement with increasing neutron energy [5]. As a consequence the neutron energy most suitable for radiotherapy would have to be determined in such a way that sufficient penetration is obtained without loss of an advantageously low oxygen effect. Our most recent experiments show, however, that the OER is very little dependent on neutron energy as shown in Fig. 7 [19]. A more detailed analysis of this phenomenon is outside the scope of this discussion. It can be concluded, however, that there is no reason to use fast neutrons with low energies and bad depth dose characteristics, but that the most suitable energy both from a practical as well as from a fundamental point of view is in excess of 10 MeV. It can be stated that for the application of fast neutrons in radiotherapy it is not necessary to compromise between depth dose characteristics and oxygen enhancement ratios, but that 15 MeV neutrons are favorable in both respects.

The discussion presented may be summarized as follows:

1. Arguments can be advanced in support of the hypothesis that the adverse late effects observed in the earlier clinical trial of fast neutron radiotherapy was at least partly due to over-exposure.

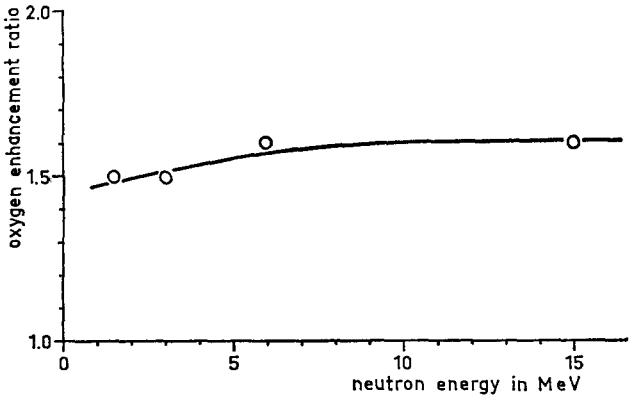

Fig. 7. Oxygen enhancement ratio as a function of neutron energy, data derived from survival curves of cultured human cells irradiated in oxygenated and anoxic conditions. Neutron sources: a: Fission spectrum neutrons, greatest intensity at 1·5 MeV, b: 3 MeV neutrons produced by the D-D reaction, c: neutrons produced by bombarding Be with 16 MeV deuterons, greatest intensity at 6 MeV, d: 15 MeV neutrons produced by the D-T reaction.

2. A possible advantage of the use of fast neutrons may be expected for those tumors which respond poorly to X- and γ-radiation, because they contain a high proportion of anoxic cells.
3. The effects of fractionation may differ for different types of normal tissues and tumors. Depending on the shapes of the relevant survival curves, the differences between effects of fast neutrons and of X- or γ-rays may vary in extent.
4. Even if fast neutron radiotherapy would turn out to be of value for only a very limited number of tumors, the results of investigations concerning fractionation effects with fast neutrons, where little recovery is observed, and with X- or γ-rays where recovery of sublethal damage is extensive, will provide a better insight in the problem of why fractionation frequently

improves the tolerance of normal tissue to a greater extent than that of the tumor and this in turn may lead to better fractionation schemes not only for fast neutrons but also for X- and γ-radiation employed in clinical radiotherapy.

RÉSUMÉ

Les résultats expérimentaux sur la guérison d'une certaine fraction de la radiolésion de cellules de mammifères montrent que ce phénomène est beaucoup moins important après l'exposition aux neutrons rapides qu'après l'irradiation aux rayons X *ou* γ. *Cette différence est liée aux variations des courbes de survie obtenues avec des irradiations de PLE (perte linéaire d'énergie) haute, par rapport à celles de PLE basse, et celle-ci influence l'effet de fractionnement.*

L'effet de l'oxygène sur la radiolésion peut être exprimé quantitativement par un facteur d'accroissement par l'oxygénation. Celui-ci a été d'une valeur approximative de 1,6 *pour les neutrons rapides et de* 2,6 *pour les rayons* X. *En conséquence de ces deux différences entre les effets des neutrons rapides et des rayons* X *ou* γ, *des variations importantes entre des tissus différents peuvent être observées en ce qui concerne l'efficacité biologique relative (EBR) des neutrons rapides par rapport aux rayons* X.

On peut montrer que l'EBR des neutrons rapides devrait être plus élevée pour les cellules en état d'anoxie — qui peuvent exister dans beaucoup de tumeurs — que pour les cellules bien oxygénées de tissus normaux. L'intérêt renouvelé pour l'utilisation des neutrons rapides dans le traitement des tumeurs a été fondé sur cet effet.

En outre, les différences dans la guérison de la radiolésion sont importantes. On s'est demandé quelle était l'énergie la plus avantageuse et on a conclu que, en ce qui concerne la qualité des doses profondes et les valeurs du facteur d'accroissement par l'oxygénation, les neutrons d'une énergie de 15 *MeV étaient les plus dignes d'intérêt.*

SUMMARY

Experimental data concerning recovery of radiation induced damage in mammalian cells show that this phenomenon is much less important after fast neutron irradiation than after X- *or* γ-*irradiation. This difference is related to differences between the shapes of survival curves obtained with high LET and low LET radiations respectively and influences the effects of fractionation. The enhancement by oxygen of radiation damage, which can be quantitatively expressed by an oxygen enhancement ratio (OER), is equal to about a factor of* 1·6 *for fast neutrons and about a factor of* 2·6 *for* X-*rays.*

As a consequence of these two differences between effects of fast neutrons and of X- *or* γ-*rays, important variations between different tissues may be found with regard to the relative biological effectiveness (RBE) of fast neutrons as compared with* X-*rays.*

For the irradiation of anoxic cells, which may be found in many tumours, it can be shown that the RBE of fast neutrons may be expected to be higher than for well oxygenated cells present in normal tisuses. This expectation is the basis of a renewed interest in the application of fast neutrons for the radiotherapy of tumours. In addition, differences in recovery may be important. A discussion is given of the neutron energy which is most suitable for this purpose and it is concluded that with respect to good depth dose characteristics as well as with regard to low OER values, 15 *MeV neutrons seem to offer the best prospects.*

ZUSAMMENFASSUNG

Experimentelle Ergebnisse betreffend die Heilung von Strahlungsschäden der Säugetierzellen haben gezeigt, dass dieser Effekt nach Bestrahlung mit schnellen Neutronen viel geringer ist als dies nach Verwendung der Röntgenstrahlen oder γ-*Strahlen der Fall ist. Diese Differenz ist auf unterschiedliche Ueberlebungskurven für Strahlungsqualitäten zurückzuführen, die durch hohen und niedrigen linearen Energieverlust charakterisiert werden können, und welche die Effekte von Fraktionierung beeinflussen.*

Die Steigerung der Strahlenempfindlichkeit durch Sauerstoff, welche quantitativ durch die Sauerstofferhöhungsratio gegeben wird, ist 1,6 *für schnelle Neutronen und* 2,6 *für*

Röntgenstrahlen. Aus diesen Differenzen der zwei Effekte für schnelle Neutronen und Röntgenstrahlen kann abgeleitet werden, dass für die relative biologische Wirksamkeit von schnellen Neutronen im Vergleich zu den Röntgenstrahlen bedeutende Unterschiede gefunden werden können.

Bezüglich der Bestrahlung von anoxischen Zellen, welche in vielen Tumoren erscheinen können, kann gezeigt werden, dass die relative biologische Wirksamkeit der schnellen Neutronen dort höher ist als in mit Sauerstoff genügend versorgten Zellen von normalen Organen. Diese Erwartung begründet das erneute Interesse in der therapeutischen Anwendung von schnellen Neutronen. Daneben ist es wahrscheinlich, dass Unterschiede bezüglich der Heilung von Strahlungsschäden wichtig sind.

Eine Diskussion der optimalen Neutronenergie zeigt, dass von 15 *MeV Neutronen bezüglich Tiefendosis und Sauerstofferhöhungsratio die besten Resultate erwartet werden können.*

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Mitotic Chromatid Separation in a Case of Acute Leukemia

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THE SEPARATION of daughter chromosomes at anaphase (a process called disjunction), is one of the most important events of normal mitosis. It can be defined as the moment when the chromatids, attached to the spindle, begin their movements towards the poles, after the splitting of the centromeres. Mitotic *non-disjunction* is one of the possible mechanisms which could account for constitutional mosaicism, as well as for a type of aneuploidy found in the nuclei of malignant tumors, namely the alteration of the total number of chromosomes without any detectable rearrangement. Indeed, the study of the normal separation of chromatids is necessary for a better understanding of the pathological processes involved in non-disjunction. The aim of the present work is to compare some observations on chromatid separation in normal cells with those in a leukemic patient, in order to contribute to a better knowledge of chromatid segregation at anaphase. However, since the present report is primarily concerned with leukemic cells, only the main points about normal anaphase are described to form a basis for comparison.

I. CHROMATID SEPARATION IN NORMAL ANAPHASE

Analysis of equatorial plates of dividing human lymphocytes from healthy donors, fixed without any pretreatment, has shown two important points:

a. The large chromosomes of the human set exhibit a "diamond-shape figure" ("figure en losange") when anaphase begins (Fig. 1). This typical figure is not surprising because the anaphasic movements involve the kinetochores, or centromeres, while other parts of the chromatids are passive [1]. The first area to separate is indeed the centromeric one, the telomeres still lying together. The diamond-shape figure is very different from the "ski figure" of the C-anaphase, in which both chromatids lie parallel to each other, after damage of the spindle by the colchicine [2].

b. The formation of the diamond-shape figures is asynchronous (Fig. 2) suggesting that anaphase begins separately for each chromosome. The whole anaphase in mammalian tissue cultures takes a few minutes [3]; the "figures en losange" are thus demonstrable during 1 or 2 min, perhaps even less. Asynchronism of chromatid separation is of very short duration and can therefore only be observed in a small number of fixed mitoses (about 2 per cent). Unfortunately, in these preparations without hypotonic treatment, identification of chromosomes is difficult and inaccurate.

II. CHROMATID SEPARATION IN LEUKEMIC CELLS

During the course of a cytogenetical survey of leukemic patients, peripheral blood and bone

marrow from a 6-year-old boy, with the clinical diagnosis of acute leukemia, were examined. At the time, the child was treated with metothrexate. Subsequently he received vincristine, and died in October 1965.

I. *Methods*

a. Peripheral blood — lymphocytes were cultured and prepared for chromosome studies according to the method of Moorhead *et al.* [4].

b. Bone marrow — 0·2 ml of bone marrow was incubated at 37°C for 15 hr in a medium composed of human serum (30 per cent) and 199 from DIFCO (70 per cent). Colcemid CIBA was added 4 hr before hypotonic treatment in 0·85% sodium citrate. Colcemid solution is prepared as follows: one ampulla of 1 mg colcemid in "solutio aquos. stabilisat". (as indicated by the manufacturer) is diluted into 100 ml distilled water, and kept for about a month at 4°C; then it is discarded and a new solution is prepared. The final concentration used to arrest mitoses is 1 μg/ml of medium. After hypotonic treatment, cells were fixed and air dried in the same way as the lymphocytes of the peripheral blood, and stained with acetic orcein.

2. *Results*

Peripheral blood — Fifty cells were counted; all had forty-six chromosomes. Ten were karyotyped: no abnormality was detected.

Bone marrow — 110 mitoses were examined. Chromosome distribution was the following:

Number of chromosomes	45	46
Number of cells	2	91
Number of cells with "loose chromatids"		17

Four types of figures could be observed.

a. Type 1 — Sixty mitoses had normal chromosomes. Ten karyotypes were also normal.

b. Type 2 — In eight mitoses the centromeric region of some chromosomes appears unusually stretched and unstained, so that both arms seem free one from the other. This feature is characteristic of a *pair* of chromosomes and is located in the same chromosome area. For example (Fig. 3) inside the group 1 of the Denver system, chromosomes no. 1 and 2 exhibit a strong narrowing of the chromatids, but not chromosome 3. For the no. 1, the constriction is juxta-centromeric while it is centromeric for chromosome 2.

c. Type 3 — In twenty-three mitoses with forty-six chromosomes, one or more chromosomes have diamond shaped figures. Both chromatids, in these chromosomes, instead of being attached together by the centromere as usual, on the contrary are separated at this position, the telomeres still retaining their usual place. The constriction described as "type 2" has not disappeared and facilitates the observation of the "figures en losange". Some chromosomes, however, have free chromatids (Fig. 4).

The karyotypes of the twenty-three mitoses of type 3 have been studied following the Denver classification. Usually homologous chromosomes show the same morphological pecularities, whatever their position in the spread metaphase. An analysis group by group makes it possible to distinguish at least two stages in the separation of chromatids, the second being an accentuation of the first.

Group I or A — During the first stage pair no. 1 shows a juxtacentromeric constriction (Fig. 5). Pair no. 2 shows a diamond shape figure; pair no. 3 exhibits a narrowing of the centromeric region. During the second stage, pair no. 1 is unchanged, pair no. 2 shows a diamond shape figure, the chromatids being more distant from each other than in the preceding period, while pair no. 3 begins to separate at the centromere.

Group II or B — First, only one of the two pairs (tentatively identified as no. 5) shows a "figure en losange" (Fig. 6). Later, both pairs exhibit the same figure, but the chromatids of pair no. 5 are almost completely separated: only the telomeres do touch each other.

Group III or C — An accurate identification is difficult in this group. However, it is possible to recognize homologous chromosomes with the criterion of the diamond shape figures (Fig. 7). For example, pairs no. 11 and 12, of the same size, differ because two chromosomes have separated chromatids and the two other have an usual shape. The longest chromosome of the group, which could not be paired (possibly the X chromosome) has formed a diamond shape in these leukemic cells only once.

Group IV or D — The diamond shaped figure is not easy to demonstrate, owing to the centromere position. At an early stage, only one pair (identified as no. 13) was found to begin chromatid separation. Later the same pair no. 13 shows an "inverted acrocentric figure": the characteristic U shape is given because the *telomeres* are still near one another, while the centromeres, the short arms and the satellites are separated. Pair no. 14 exhibits a clear lozenge figure, and pair no. 15 shows only the constriction of the centromeric region, giving to the short arms an appearance of enlarged satellites (Fig. 8).

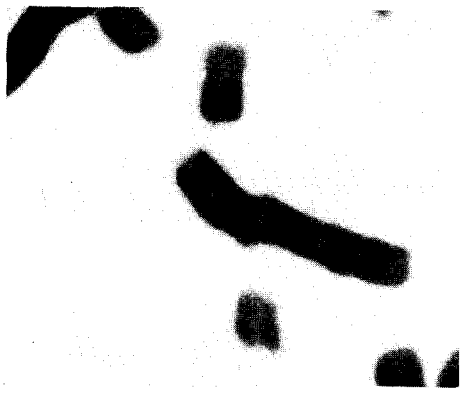

Fig. 1

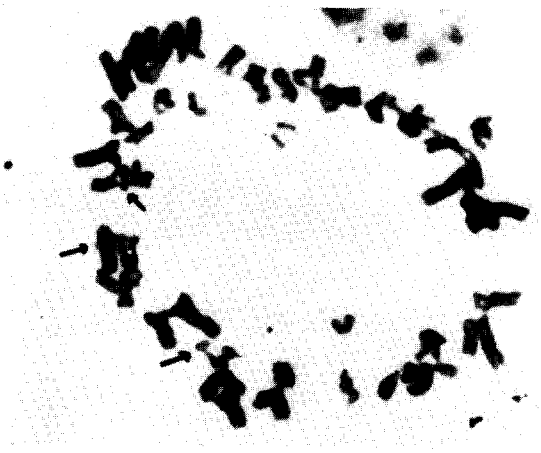

Fig. 2

Fig. 3

Fig. 1. *The onset of anaphasic movements in a large human chromosome. The centromeric region is the first to separate into two, giving a "diamond shape figure".*

Fig. 2. *Equatorial plate from a normal donor. Arrows point some disjoined chromatids. It may be seen that all chromosomes are not at the same stage.*

Fig. 3. *Juxta-centromeric constrictions in group A. The site of this constriction is not the same for each pair.*

(to face p. 348)

Fig. 4. Well spread metaphase from bone-marrow of a leukemic patient. Asynchronization of chromatid separation is demonstrated by the presence of clear diamond shaped figures while other chromosomes retain their usual X appearance.

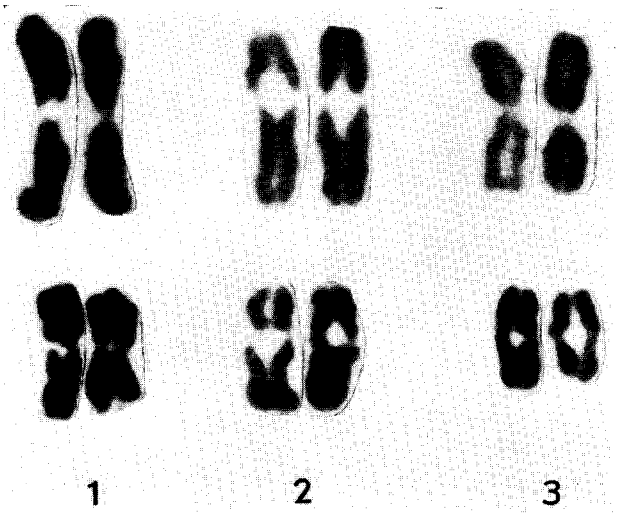

Fig. 5

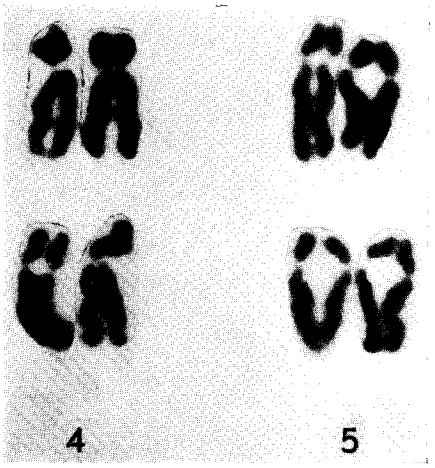

Fig 6

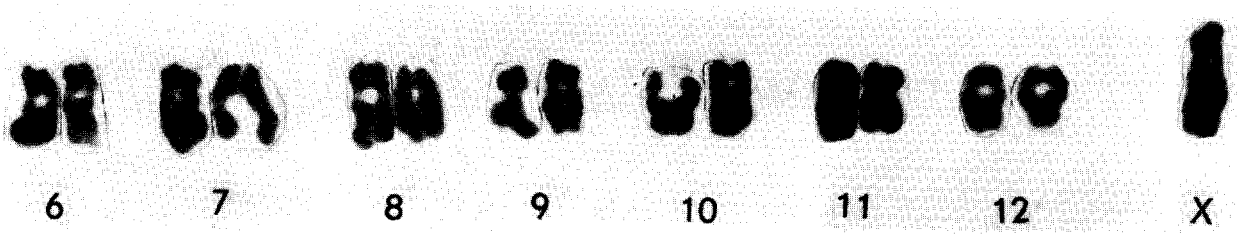

Fig. 7

Fig. 8

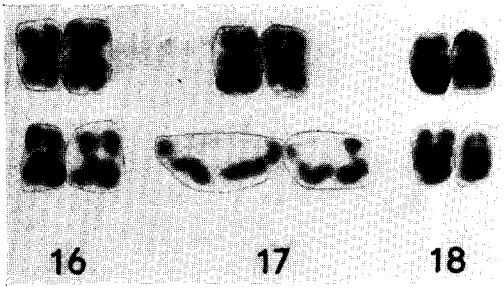

Fig. 9

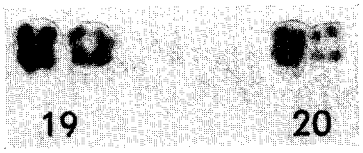

Fig. 10

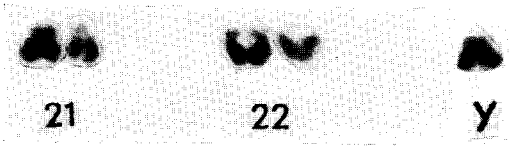

Fig. 11

Fig. 5. *Chromosomes of the group A. Early stage above, later stage below. Chromosome* 3 *begins its centromeric separation before chromosome* 1 *and after chromosome* 2.

Fig. 6. *Group B. During the early stage, only pair no.* 5 *exhibits a diamond shaped figure. Later, this figure is accentuated and appears in chromosome* 4.

Fig. 7. *Group C. A tentative of classification has been made with the criterion of centromeric separation. Pairs of the same size may differ by the onset of anaphase movements (see for example pairs no.* 11 *and* 12).

Fig. 8. *Group D. At a late stage, the "inverted acrocentric figure" can be observed. The short arms of chromosome* 15 *are similar to enlarged satellites.*

Fig. 9. *Group E. The chromosome no.* 17 *begins its centromeric separation very early. When no.* 18 *and* 16 *exhibit juxta-centromeric constrictions or diamond shape figures, chromatids of no.* 17 *are almost completely separated.*

Fig. 10. *Group F. The chromosomes are short, so chromatid separation is difficult to observe. However, a diamond shape figure may be distinguished in pair* 19.

Fig. 11. *Group G. An "inverted acrocentric figure" appears in no.* 22, *while no.* 21 *and chromosome Y have their usual appearance.*

Group V or E — As in group D, the three pairs have a different behaviour . During the first stage, only pair no. 17 begins to disjoin. Later, chromosome 16 presents a strong narrowing of the centromeric region, pair no. 18 begins to disjoin, while the chromatids of both chromosomes 17 are almost completely dissociated (Fig. 9).

Group VI or F — It is difficult to distinguish two stages in this group, very likely because of their shortness. Nevertheless, one pair (tentatively identified as no. 19) exhibits a diamond shape figure before the other (Fig. 10).

Group VII or G — In this group again two stages are difficult to distinguish. However, one pair, (no. 22) has an "inverted acrocentric figure" similar to chromosome 13 in the late stage, while no. 21 and the chromosome Y are still unchanged (Fig. 11).

An attempt has been made to observe the frequence of the diamond shape figures for each pair in the twenty-three mitoses available. Chromosome 17 exhibits diamond shape figures in nearly all mitoses (Table 1), followed by pairs no. 5, 2 and 13. In the group C, nos. 6 and 11 have the greatest number of diamond shape figures, however less frequently than the four pairs already mentioned. The remainder of group C, except the largest (the presumptive X) resembled chromosomes nos. 3, 19, 20 and 22 by exhibiting separated chromatids in almost the same number of mitoses. Chromosomes 1, 4 and 14 show lozenge figures in only three or four mitoses. Chromosomes 15, 16, 18, 21, X and Y show disjoined chromatids very seldom, if any.

d. Type 4 — The ninety-two chromatids are separated from each other (Fig. 12), as observed in seventeen cells.

3. *Discussion*

The comparison with the figures observed in the normal makes it very likely that in the case of the leukemic child presented here the function of the spindle has not been disturbed by colchicine, for an unknown reason. It would have been important to know exactly when the colcemid stock solution used in this case was prepared, for its activity may decrease with time. Unfortunately, this was not recorded. However, the absence of response to colcemid has never been observed in other cultures from this laboratory. Moreover, all cultures arrested 2 weeks before and 2 weeks after the present had mitoses with the usual appearance after colcemid treatment; some of these cultures were certainly arrested with the same solution used in the leukemic case presented here. In this culture, chromatid movements seem to have been stopped only by the hypotonic treatment. After the spreading process, all mitotic structures were disorganized; but then the chromosomes could easily be identified. Until now, all attempts to reproduce similar figures in this laboratory have failed, especially in cells treated in hypotonic medium for 20 min without colchicine. So it must be stressed that this is a very special case. Thus the following discussion concerns mainly the separation of chromatids in apparently normal karyotypes in both blood and bone marrow cells of a leukemic patient. However, reference to the normal shall be considered, although a generalization of the results is not possible for the moment.

Table 1

Chromosome pairs	1	2	3	4	5	6	7	8	9	10	11	12
Number of karyotypes having disjunction	3	15	7	4	20	10	7	9	7	6	10	6

Chromosome pairs	13	14	15	16	17	18	19	20	21	22	X	Y
Number of karyotypes having disjunction	13	3	0	1	22	1	6	6	1	6	1	0

Diamond shape figures have been counted for each chromosome through twenty-three mitoses. The total number of these figures (quoted in the table as "disjunction") are reported below the chromosome number (Denver system).

a. Constriction of the centromeric region — The first sign of the chromatid separation seems to be the narrowing of the centromeric region, i.e. the area proximal to the spindle attachment. The first chromosomes to present this elongated area are the first to show separated chromatids and diamond shape figures: both mechanisms appear to be linked. For some chromosomes, especially for nos. 1, 9 and 16, the narrowing of the juxta-centromeric region is identical to the secondary constrictions already described [5,6] Thus the possible origin of secondary *juxta-centromeric* constrictions could be found in the onset of anaphasic movements. A mechanical action, the "pulling" of the kinetochore on the passive chromatid, would produce an elongation of the proximal area.

b. The asynchronous onset of anaphasic movements — The asynchronization of chromatid migration towards the poles has undoubtedly been observed (Fig. 2). Mitotic figures observed in normal cells have also been seen in this unusually clear case of a leukemic child with normal chromosomes. It must be stressed that usually in the same mitosis *both* chromosomes of the same pair exhibit at the same time the same degree of chromatid separation. Anaphase begins at the same moment in both members of a pair of chromosomes, with very small variations. This demonstrates their homologous behavior, whatever their situation on the spindle. The first chromosomes to move towards the poles are indeed those presenting the more frequently separated chromatids through different mitoses. In the present case, pairs nos. 17, 2, 5 and 13 migrate first, followed by nos. 11, 8, 6, 7, 9, 10, 20, 3, 12, 22, 4 and 1. The last chromosomes to migrate are those with only one or without any diamond shape figures, that is to say nos. 14, 15, 16, 18, 21, X and Y. Surprisingly enough, it may be noticed that, except for chromosomes 16, the latest migrating chromosomes are those most usually involved in human trisomies [7], as well as in aneuploidies such as those found in some malignant cells. A "slow down" of physiological processes could then be the cause of aneuploidies not involving morphological rearrangements. Inversely, an acceleration of telophase could affect one or more chromosomes still near the equator of the cell, and produce aneuploid cells. However, it is only possible to present such *hypothesis* about purely mechanical causes of aneuploidies, on the basis of observations in one leukemic patient, presented here.

c. Correlation between DNA synthesis and anaphase movements — It is now well known that in man DNA synthesis is asynchronous at the chromosome level [8–13]. Inside the groups, chromosomes sometimes difficult to identify are easily recognized after radiothymidine incorporation and autoradiography. Group D is the best example for human *autosomes:* the three pairs of this group are morphologically very similar; accurate measurements are not always adequate for a correct identification. However, after tritiated thymidine incorporation, it is possible to distinguish a pair completing the DNA synthesis early during the S period, a second pair achieving DNA synthesis some time later, and a third pair labelled very late and usually involved in cases of trisomy D [14]. The present study of chromatid disjunction in a case of acute leukemia also shows 3 different kinds of behaviour in group D: one pair moves the first to the poles, followed soon after by a second pair, the third one migrating very late, In the group C, the example of the late labelled X in the normal female is well known, and also the multiplicity of aneuploidies involving the X chromosome. The present case concerns a male subject, without late labelled X. However, if identification was correct, the X chromosome presented only once a diamond shape appearance. Chromosome Y never showed any signs of centromeric separation. These facts suggest that the sexual chromosomes in male diploid cells are late migrators. In group B, chromosome 4 is late labelled over all its length; at the same moment, chromosome 5 has only heavy labelled short arms [15]. Anaphase movements begin later for chromosome 4. Chromosome 17, of group E, is generally considered as the first autosome of the human set completing DNA synthesis; it is also the first to show separated chromatids. Chromosomes 16 and 18, of the same group, are late labelled and late in separating their chromatids.

Emphasis must again be laid on the fact that general conclusions about anaphase in human cells cannot be drawn from the present study of separation in a leukemic patient. Work is in progress actually in this laboratory to determine if *in the normal* the latest labelled chromosomes are also the latest migrators towards the poles. Since the frequency of diamond shape figures is very low (2 per cent of all mitoses in fixed preparations of lymphocytes without any pretreatment) compared to the frequency of anaphases (11 per cent), observations shall be mainly based on anaphase. In the present case, the sequence of chromatid separation at anaphase appears to be intimately correlated with

the published sequence of DNA synthesis among chromosomes in the human karyotype. The later a chromosome completes its DNA synthesis, the later this chromosome begins its anaphasic movements, and the greater are its chances to be involved in non-disjunctive aneuploidies. Thus the origin of non-disjunction could be found *before* the onset of mitosis. Ohno et al. [16] have already suggested that the nucleolus could be the cause of non-disjunction of the nucleolus-organizer chromosomes. Since no RNA synthesis is detected after mid-prophase [3], the origin of this type of non-disjunction should also be found *before* the onset of active mitotic processes.

4. *Conclusions*

a. The study of mitotic figures in normal dividing lymphocytes has shown, as expected, that separation of chromatids at anaphase begins at the kinetochore. A constriction of the centromeric region and typical "diamond shape figures" are observed just after the onset of the movement.

b. Onset of anaphase in normal cells is asynchronous: some chromosomes have diamond shape figures, while others do not.

c. Diamond shaped figures have been observed in bone marrow cells from a case of acute leukemia. For an unknown reason, the spindle function was not impaired by colchicine treatment.

d. Asynchronism was the rule in this case, as in the normal. Classification following the Denver system has shown that homologous chromosomes begin anaphase at the same moment.

e. The later a chromosome completes its DNA synthesis, the later it migrates towards the poles. A correlation between DNA synthesis, mitotic processes and aneuploidy is suggested. However, this suggestion is restricted, for the moment, to the present case.

RÉSUMÉ

L'étude de la disjonction des chromatides à l'anaphase a montré, chez le normal, que le début des mouvements ascensionnels est caractérisé morphologiquement par l'apparition "d'images en losange". Ces images, qui traduisent l'action mécanique des kinétochores sur les chromatides passives, ont été retrouvées dans les cellules de la moelle osseuse d'un sujet atteint de leucémie aig Huë. Le classement des chromosomes dans 23 mitoses a permis de constater que l'ascension vers les poles est asynchrone. Il semble exister une corrélation entre la fin de la synthèse d'ADN et la migration anaphasique: plus tard un chromosome termine sa période de synthèse, plus tard il migrera vers les poles. L'importance de ce phénomène dans la genèse d'aneuploïdies par "non-disjonction" est discutée. Mais seule l'étude ultérieure de mitoses normales permettra de trancher cette question.

SUMMARY

The study of the anaphase chromatid separation in normal lymphocytes showed that the onset of the movement is asynchronous and morphologically characterised by "diamond shape figures", very likely due to the pulling action of the centromeres on the passive chromatids. These typical figures have been observed in a case of human acute leukemia, in bone marrow cells treated with colchicine and hypotonic medium. It has been possible to identify the chromosomes. A correlation between DNA synthesis and anaphase migration is suggested: the later a chromosome duplicates, the later its chromatids separate and move towards the poles. This suggestion however is only volid for the present case, and cannot actually be generalized.

ZUSAMMENFASSUNG

In normalen Lymphocyten hat das Studium der Chromosomentrennung, die während der Anaphase stattfindet, gezeigt dass der Anfang der Wanderung asynchron verläuft und durch das Erscheinen von spindelförmigen Bildern morphologisch charakterisiert ist. Diese Bilder, welche der Zugwirkung der Kinetochoren über die passiv gebliebenen Chromosomen entsprechen, wurden auch in den mit Colchicin und in hypotoner Lösung behandelten Knochenmarkszellen eines an akuter Leukämie leidenden Krankes gefunden. Die Chromosomen konnten identifiziert werden. Zwischen der DRNS-Synthese und der anaphasischen Wanderung scheint eine Wechselwirkung zu bestehen; je spater ein Chromosom sich spaltet, desto später trennen sich Tochterchromosomen und wandern nach den Polen.

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Demonstration of Virus Particles in Biopsy Material from Cases of Burkitt's Tumour

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INTRODUCTION

THE CLINICAL and pathological features of Burkitt's tumour have been fully described [1, 2] and the circumstantial evidence which lead to the suggestion that it might be caused by an arthropod-borne virus has been comprehensively reviewed [3, 4]. Harris [4] concluded "that the tumour probably results from a bizarre host reaction towards a common arthropod-borne virus or group of arthropod-borne viruses".

A variety of agents have been isolated from biopsy material from cases of Burkitt's tumour [5]. Herpes simplex, reovirus type 3 and mycoplasma have all been recovered in mice or tissue culture; while a herpes-like agent has been demonstrated in cultures of tumour cells. So far no reports of the observation of virus particles directly in tumour tissue have been made. It might be expected that if such agents as reoviruses and herpesviruses can be demonstrated following tissue culture manipulative techniques, then they should also occasionally be found by direct methods. In the absence of such direct evidence, it always remains a possibility that the agents are contaminants, not necessarily from the laboratory, but from the patients themselves. This possibility is certainly more likely for the isolates made from jaw tumour biopsies which may be contaminated by buccal mucosa or saliva and is much less likely for those isolations made from biopsies of non jaw lesions.

This report describes the demonstration of both herpesvirus- and reovirus-like particles by direct electron microscopy of biopsies of Burkitt's tumour.

MATERIALS AND METHODS

Examination of biopsies

Fragments of biopsy material collected in the operating theatre were immediately immersed in 2·5% buffered glutaraldehyde for 12–18 hr, post-osmicated in 1% osmium tetroxide, dehydrated in graded alcohol and embedded in araldite. On a Reichert ultramicrotome, thick

(0·5μ) sections were cut, and stained with toluidine blue-pyronin [6] for light microscopy. Thin sections for electron microscopy were mounted onto Athene 483 grids without a supporting membrane, stained with uranyl acetate and examined in a Zeiss E.M.9 microscope.

Six specimens were prepared for direct examination using a Spinco L50 ultracentrifuge. Three were biopsies of ovarian tumours previously identified histologically as Burkitt's tumour, one a mouse brain infected with herpes simplex virus*, one a reovirus-infected tissue culture of vervet monkey kidney cells, and as a control, a normal tissue culture of vervet monkey kidney cells. Each biopsy was homogenized for 2 min in an M.S.E. homogenizer and then frozen overnight in a mechanical deep freeze at −60°C. Both the homogenates and the tissue cultures were stored at −60°C until centrifuged. All six preparations were processed simultaneously as follows. The cell debris was removed by centrifuging at 5000 rev/min in a Servall R.C.2 centrifuge, and the supernatant then spun in a '50' head in the Spinco L50 at 20,000; 30,000; 40,000 and 50,000 rev/min. The deposit was collected at each stage, and the supernatant transferred to be spun at the next higher speed. At each step 1 hr was allowed for centrifugation after the required speed had been reached.

The deposits were stored at −60°C until needed for examination in the electron microscope. Directly before examination the specimens were allowed to warm to room temperature. One drop of the deposit (taken from the tube with a 2-mm platinum loop) was placed on a formvar coated Athene 483 grid gripped in forceps, left for 10 sec, and washed off with 0·1M calcium chloride. A drop of 2% phosphotungstic acid (buffered to pH 7·3) was then placed on the grid, left for 5 sec and the grid touched to a lint-free tissue to remove the fluid. The grid was then stored over anhydrous calcium chloride until examined.

Mouse brain experiments

J262 — Part of a maxillary biopsy was minced in a 50: 50 mixture of medium L [8] containing 100 mg penicillin, and streptomycin, and guinea pig serum (inactivated at 58°C for 30 min). Aliquots of 0·1 ml of this suspension of tumour cells were injected intracerebrally into each of seven newborn white Swiss mice, subsequently given 1·0 mg cortisone acetate intraperitoneally on alternate days. The mice died 3, 4, 7, 8 and 14 days after inoculation. The brain from one survivor at 14 days was minced in medium L and guinea pig serum and 0·1 ml of this suspension injected into the brains of eight cortisone-treated newborn mice. This suspension contained occasional Burkitt tumour cells. The brain from one survivor of the second litter at 5 days was minced and fixed in glutaraldehyde.

K231 — A large pleural effusion in a 12 year-old boy was aspirated and found to contain large numbers of Burkitt tumour cells. These were concentrated by centrifugation and 0·1 ml of the deposit was injected into the brains of each of ten newborn mice. All the mice survived. One animal was sacrificed after 7 days and the brain fixed in glutaraldehyde.

Both mouse brain specimens were subsequently treated in the same way as the biopsy specimens.

Tissue cultures

Monolayers of vervet monkey cells were infected with the CAN 220, CAN 228 and CAN 230 strains described in detail elsewhere [8]. When a 50 per cent cytopathic effect was observed, the cells were removed from the glass using 0·25% trypsin (Difco 1: 250) in Hanks' B.S.S., centrifuged and packed into a small pellet, which was cut into several pieces and prepared for examination in the same way as the biopsy specimens.

RESULTS

Five biopsy specimens have been examined by thin sectioning. The first, CAN 309, was a tumour of the left maxilla; the next three were all ovarian tumours; and the last a biopsy from a massive breast tumour. In four of the specimens, particles were demonstrated, whilst in the fifth (CAN 323 — ovarian tumour) no particles were found despite all six fragments being sectioned at several levels. This particular specimen will be referred to again when the results obtained by ultracentrifugation of the tumour tissues are discussed. In the maxillary biopsy some thirty cells were seen containing numerous intranuclear particles out of a total of approximately 12,000 cells examined. The particles are morphologically similar to those obtained first by Epstein *et al.* [9] and later by Stewart *et al.* [10] in tissue culture. They tend to appear hexagonal with an eccentric nucleoid, and show a size variation within the range 75–100 mμ (Figs. 1–4). In the other specimens, far fewer infected cells were found (1 in 1,500 to

*Infected [7] with strain CAN 63/13 (111337–348) and kindly supplied by Mr. M. Lule of the East African Virus Research Institute.

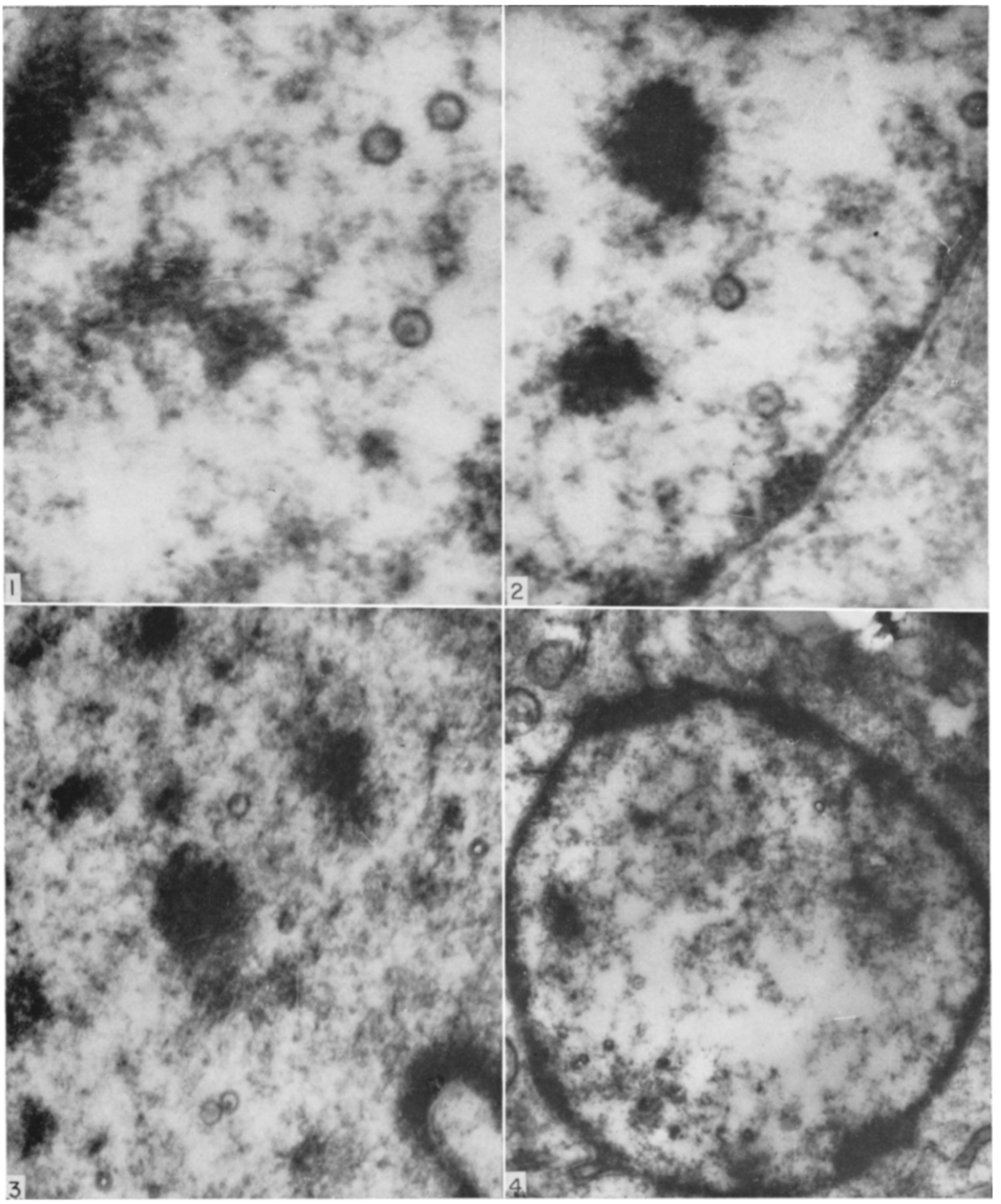

Fig. 1. *Biopsy of maxilla, CAN* 309 (*J*248). *Three herpes-like intranuclear particles* (~82 mμ). *Uranyl acetate stained.* ×68,000

Fig. 2. *Infected nucleus from cell of CAN* 309 (*J*248); *similar particles to Fig.* 1. *Uranyl acetate stained.* ×68,000.

Fig. 3. *CAN* 309 (*J*248) *Intranuclear particles, some with double membrane, others with distinct nucleoid* (85mμ). ×38,400.

Fig. 4. *CAN* 309 (*J*248) *Intranuclear particles* (85 mμ). ×18,200.

(to face p. 354)

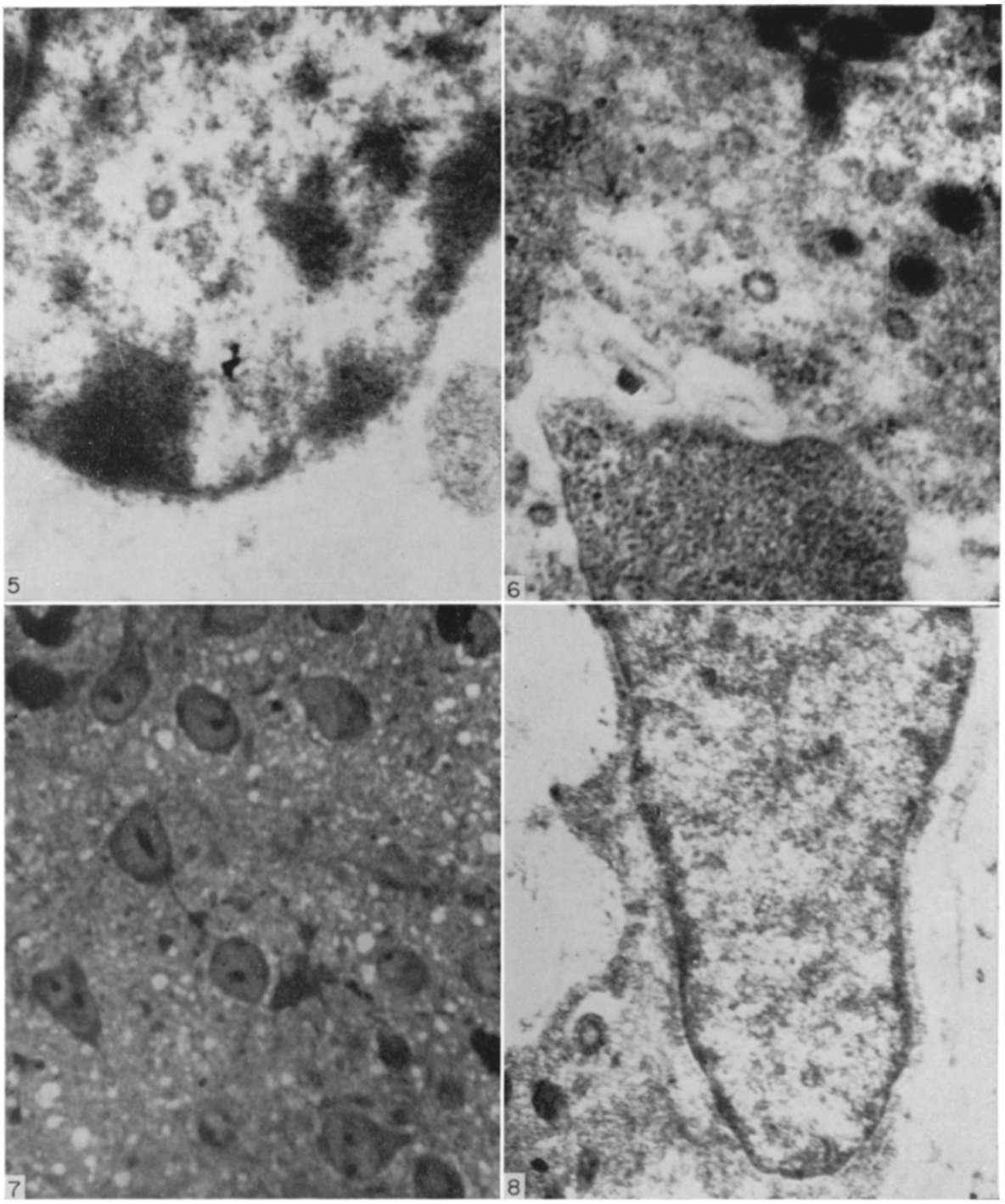

Fig. 5. *Biopsy of ovarian tumour, CAN* 371 (*J*264). *Nuclear particle* (145 *m*μ). ×25,200.
Fig. 6. *Biopsy of breast tumour,* K232 (*B*5579/65). *Cytoplasmic particles* (95 *m*μ). ×57,600.
Fig. 7. *Mouse brain inoculated with pleural fluid containing Burkitt tumour cells, K*231 (*B*4601/65)· *Light microscope photograph of area selected for electron microscopic study. Toluidine blue/pyronin.* ×1120.
Fig. 8. *K*231 (*B*4601/65) *Double-walled oval cytoplasmic particle* (160×220 *m*μ). ×24,000.

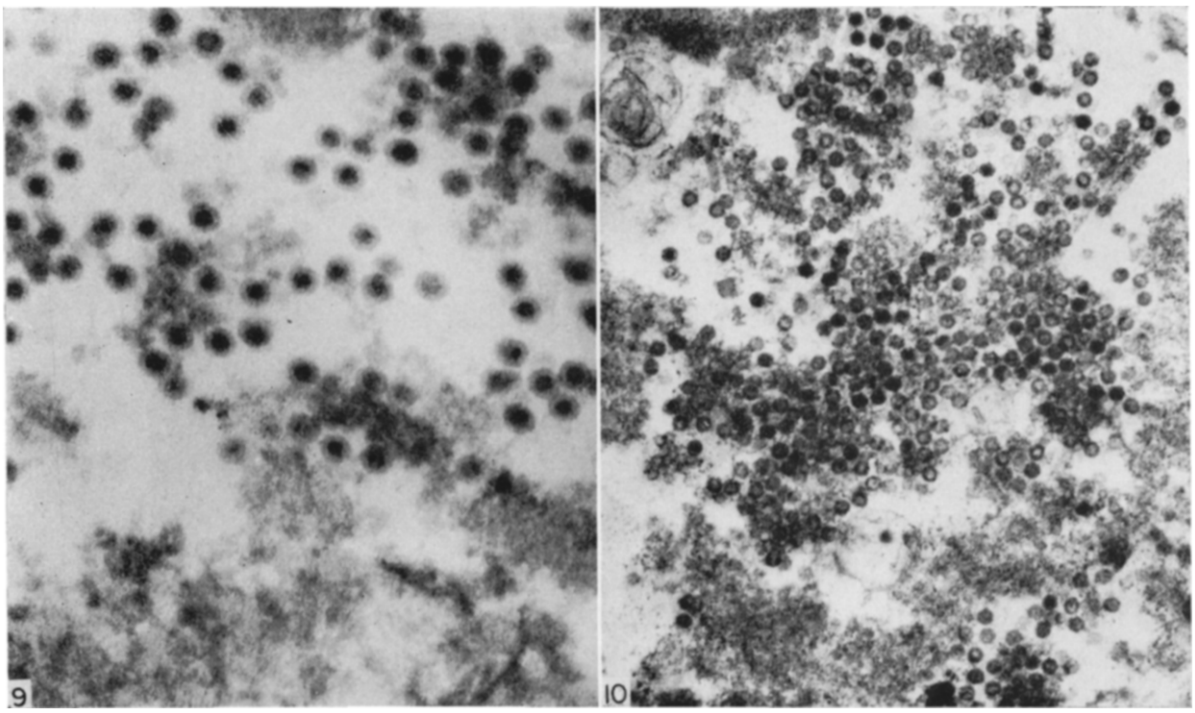

Fig. 9. *Vervet monkey kidney cells infected with CAN* 230 *strain of reovirus type* 3 (69 mμ). *Uranyl acetate stained.* × 57,600.

Fig. 10. *Vervet monkey kidney cells with unidentified virus from CAN* 220 (54 mμ). *Uranyl acetate stained.* × 29,200.

1 in 2,500), and the majority of the particles were observed in the cytoplasm (Fig. 6). The cytoplasmic particles are thick-walled, oval particles, with an average diameter of 85 mμ, and are morphologically similar to, although significantly larger than, the reovirus strains obtained in tissue cultures. Occasional intranuclear particles were also found (Fig. 5).

From the specimens examined following ultracentrifugation, no particles were observed in the normal tissue culture cell suspensions, nor in the suspensions from CAN 262 (ovarian tumour), although three cells were found in direct tissue microscopy containing a small number of cytoplasmic particles. From all the other suspensions (including CAN 323 which was negative on direct microscopy) a small number of particles was seen after centrifugation at 20,000 rev/min, a moderate number after 30,000 and 40,000 rev/min and none at 50,000 rev/min. The particles from the CAN 230 infected cells are similar to reovirus type 3 similar to complete herpesvirus particles. In the tissue culture grown virus cultures (CAN 228, CAN 230 and CAN 220), particles morphologically resembling reovirus type 3 were seen (Figs. 9 and 10). These particles have been discussed elsewhere [3, 6].

The full results are summarized in Table 1.

DISCUSSION

In four of the five biopsies examined by direct thin-sectioning techniques, particles similar to the internal component of a herpesvirus have been observed. From one of these, J248, two of us have independently isolated herpes simplex virus from different pieces of biopsy material, in mice (D.H.W.), and in tissue culture (M.G.R.R.). It is therefore probable that the particles observed in thin sections of this tumour are, in fact, herpes simplex virus. The frequency of observation of infected cells is much higher with this tumour than with the others (1 in 400 compared with 1 in 1,500 to 2,500). The remaining three are therefore probably similar to the agents described by Epstein [11] and Stewart [13] in tissue cultures of Burkitt's tumour.

Table 1. *Demonstration of virus particles in seven cases of Burkitt's tumour*

Case No.	Direct biopsy	Following ultra-centri-fugation	Following inocula-tion into mouse brains	Type of particle	Site	Size (mμ)
CAN 309 (J248)	+	N	N	Herpes-like	Nucleus	75–100
CAN 262 (J232)	+	—	N	Herpes-like	Cytoplasm	~85
CAN 323 (J260)	—	+	N	Herpes-like	oo	~85
CAN 371 (J264)	+	+	N	Herpes-like	Cytoplasm	
					Nucleus	~85
				Reovirus-like	oo	~60
— (K232)	+	N	N	Herpes-like	Cytoplasm	80–90
CAN 325 (J262)	N	N	+	Herpes-like	Cytoplasm	~90
— (K231)	N	N	+	Large double-membraned	Cytoplasm	~200

N=method not used.

oo=not applicable; virus only seen after ultracentrifugation.

control (prepared earlier), and the particles from the ovarian tumours to the herpes simplex control. In CAN 371 (ovarian tumour) particles resembling reovirus were seen in addition to those resembling herpes simplex virus, and reovirus type 3 was isolated from this tumour.

Cells containing cytoplasmic particles were found in both of the specimens of infected mouse brain. Those in the mouse infected with J 262 were similar to those found in the direct biopsy material, while those in the mouse infected with pleural fluid from K231 (Fig. 7) were larger, 200 mμ, oval, double-walled particles, (Fig. 8)

The demonstration of herpesvirus-like and reovirus-like particles by direct examination of tissue from cases of Burkitt's tumour indicates that these two viruses are present in the tumour and have not been "picked up" as laboratory contaminants. The herpesviruses have been observed both in thin sections and centrifuged material, but the reoviruses only in centrifuged material. The herpes-like particles have been observed in cultures of Burkitt's tumour cells

[11, 12], in cultures of acute myelogenous leukaemia cells [12], and in a reovirus-associated murine lymphoma [13]. Reoviruses have been isolated from cases of Burkitt's tumour [8] and are associated with a murine lymphoma that has several similarities to Burkitt's tumour [14].

Three strains of virus have been detected in mouse brain passage. One, CAN 309, seen in direct examination of tumour tissue (J248), was also detected in tissue culture and found to be herpes simplex virus, while the other two did not pass serially and were only observed in the electron microscope. They appear to be similar to the SL_1 virus of Stewart [12].

In biopsy specimens of Burkitt's tumour few, if any, complete herpesvirus particles have been seen. Smith [15] has shown that the outer coat appears to be necessary for high infectivity. If the virulence of the virus has also been reduced, as occurs with latent infections of rabies virus in rabbit endothelial cells [16], this could explain the difficulty in demonstrating biological activity of the SL_1 and other strains.

The question of the possible role of herpesviruses and reoviruses in the aetiology of Burkitt's tumour remains unanswered. Latent herpesvirus infections are well known, and transplacental infection has been demonstrated for herpes simplex [17] and a canine herpesvirus [18]. The Lucké virus is associated with renal tumours of frogs [19], while chromosome alterations can be induced by herpes simplex [20]. Herpes simplex virus has also been shown to increase the incidence of tumours produced by chemical carcinogens [21]. However, no member of the herpesvirus group has yet been shown to be arthropod vectored, and particles similar to those seen in Burkitt's lymphoma have been seen in the reovirus-associated murine lymphoma [13]. Reoviruses can also give rise to latent infections [22] and replication occurs in close association with the mitotic apparatus of the host cell [23]. They can be arthropod vectored [24], and the closely related plant reovirus, wound tumour virus, is known to be transmitted only by insects [25]. Despite the different nucleic acids, herpes and reovirus fall into the same, apparently distinct, group on the basis of sensitivity to ultraviolet light [26].

There are several possible explanations for the observations that herpesviruses and reoviruses are associated with Burkitt's tumour. Either or both may be latent infections and have no direct connection with the tumour. Conversely, either or both may be the aetiological agents of the disease. It is possible that one is the analogue of the other, and given the right conditions they may together induce neoplastic transformation.

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RÉSUMÉ

Des échantillons de tissu tumoral de 7 cas de tumeur de Burkitt ont été examinés au microscope électronique. Cinq biopsies furent examinées directement, trois d'entre elles après ultracentrifugation, et 2 autres après traitement et injection dans le cerveau de souris suisses nouveau-nées. Des particules ressemblant au virus d'herpès ont été distinguées dans tous les échantillons de tissu, au moins avec une des méthodes utilisées, alors que le réovirus a été vu dans un cas, après ultracentrifugation. On peut conclure de ces constatations, en y ajoutant d'autres résultats rapportés, que le virus d'herpès et le réovirus sont tous deux associés à la tumeur de Burkitt, bien que leur rôle dans la maladie soit encore incertain.

SUMMARY

Tumour tissues from seven cases of Burkitt's tumour have been examined by electron microscopy. Five biopsies were examined directly, three of these following ultracentrifugation and two others after treatment and injection into the brains of newborn Swiss mice. Herpesvirus-like particles have been demonstrated in all the tissues by at least one of the methods used, while reovirus has been demonstrated in one following ultracentrifugation. In conjunction with other reported evidence it is concluded that both herpesvirus and reovirus are associated with Burkitt's tumour, although their role in the disease is still uncertain.

ZUSAMMENFASSUNG

Tumorgewebe von sieben Burkitt-Tumorsfällen wurde elektronenmikroskopisch untersucht. Fünf Biopsien wurden sofort geprüft, drei davon wurden nach Ultrazentrifugierung wieder untersucht, und zwei anderen nach Behandlung und Injektion in das Gehirn von neugeborenen Schweizerischen Mäusen. Partikel, die dem Herpesvirus gleichen, wurden durch mindestens eine der verwendeten Methoden in allen Geweben erkannt. Das Reovirus dagegen wurde nur in einer nach Ultrazentrifugierung geprüften Biopsie erkannt. Diese Tatsachen, zusammen mit anderen angegebenen Ergebnissen, leiten zu der Schlussfolgerung, dass das Herpesvirus und das Reovirus mit dem Burkitt-Tumor verbunden sind, obwohl ihre Rolle in der Krankheit noch unsicher sei.

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Les Substances Antiglobulines Humaines et Anti-Gm Dans le Sérum des Cancéreux

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L'IMPORTANCE des gamma-globulines humaines en physiopathologie explique suffisamment le grand intérêt porté à la connaissance des groupes sériques.

Le polymorphisme de ces groupes, dont la spécificité a été démontrée par plusieurs auteurs, a donné lieu à une nouvelle classification des gamma-globulines. Deux d'entre eux, les systèmes Gm et Inv, génétiquement contrôlés, ont été particulièrement étudiés. Ces deux systèmes ont des supports moléculaires différents. Les facteurs du système Gm sont localisés uniquement dans les gamma-globulines (7S) IgG, tandis que ceux du système Inv se trouvent dans toutes les fractions des gamma-globulines IgG, IgM, IgA et dans les protéines de Bence-Jones.

Les déterminants des facteurs du système Gm se trouvent dans le fragment B (fraction Fast) et sur la chaîne lourde A ou H, tandis que les déterminants des facteurs du système Inv ont été mis en évidence dans les fragments A, C (fraction Slow) sur la chaîne B ou L. [1–3].

Le nombre des facteurs du système Gm, qui ne comprenait au début que trois facteurs Gm (a), Gm (b), Gm (x), s'élève actuellement à huit [4–6].

Le rôle des groupes sériques, dans la physiopathologie humaine, n'est pas encore entièrement élucidé. Des modifications de la fréquence de certains groupes Gm ont été signalées dans divers états pathologiques: myélomes [7–10], leucoses lymphoïdes et aiguës, et cancers [11–13].

Un affaiblissement du facteur Gm (a), qui peut aboutir à la disparition complète de ce facteur, au cours de l'évolution de la maladie, a été observé chez certains malades atteints de leucose [14].

Quelques observations pourraient plaider en faveur du rôle des groupes sériques dans le choix de donneurs de greffons d'organes [5, 15].

L'étude des antigènes des groupes sériques Gm ou Inv a pu exceptionnellement être réalisée à l'aide d'hétéro-immunsérums provenant d'animaux immunisés (singe, chèvre) [16–18]. Dans le sérum des sujets normaux, on peut, dans un pourcentage de cas relativement faible, compris entre 0,33% et 1,26%, observer la présence d'anticorps, naturels semble-t-il, permettant de déceler le facteur Gm. La fréquence de ces anticorps est

*Attaché de Recherches au C.N.R-S.

beaucoup plus élevée au cours de la polyarthrite chronique évolutive, où elle atteint 10%, et chez les sujets âgés de plus de 70 ans. Ces anticorps s'observent aussi chez les polytransfusés, et notamment chez des enfants.

Le mécanisme de leur apparition est encore mal défini; on peut, pour certains cas, évoquer une iso-immunisation, notamment chez les polytransfusés, ou, en cas d'injection de gammaglobuline, ainsi que chez les enfants présentant une incompatibilité de groupe sérique avec leur mère. Dans d'autres cas, on ne peut que soulever l'hypothèse d'une auto-immunisation lorsque l'anticorps anti-Gm est spécifique d'un facteur Gm que possède le malade.

Il était important de vérifier, chez les cancéreux qui présentent assez souvent certains types plus ou moins évidents de dysprotéinémie, la fréquence des substances antiglobuliniques ou anti-Gm et de comparer la spécificité de ces dernières avec le phénotype Gm possédé par le malade.

MATERIEL ET METHODES

Les sérums étudiés proviennent de malades atteints de tumeurs malignes de diverses localisations. Comme témoins, on a utilisé les sérums de sujets malades non cancéreux. En tout, les sérums de 1.363 cancéreux et 228 non-cancéreux ont été examinés.

Les groupes sériques Gm (a), (b), (x) d'une grande partie de ces sérums ont été établis dans un travail précédent [13].

Tous les sérums ont été prélevés à jeun et conservés à une température de −20°. Les examens ont été effectués, à plusieurs reprises, chez les mêmes malades, avant le traitement, et au cours de l'évolution de la maladie.

Nous avons recherché successivement:

(1) Les groupes sériques Gm (a), (b), (x) par la méthode d'inhibition.

(2) Les substances anti-gamma globuliniques et leur spécificité dans le système Gm. (Pour les détails techniques sur ces méthodes nécessitant des réactifs sélectionnés, voir [18]).

(3) Les facteurs rhumatoïdes classiques par la réaction de Waaler Rose (modifiée) et le test au Latex.

RESULTATS

La première partie de cette étude concerne les facteurs sériques Gm (a), Gm (b), Gm (x). Onze cent cinquante-quatre sérums de malades atteints de tumeurs malignes et 209 sérums de malades non cancéreux ont été examinés, 540 en ce qui concerne le facteur Gm (a) seulement. La fréquence trouvée de ce phénotype est statistiquement identique à celle observée chez les malades non cancéreux et chez les sujets normaux (Tableau 1). Il en est de même quand il s'agit des divers phénotypes en fonction de la localisation des tumeurs, de l'histologie, et de l'âge des malades. Par contre, il est intéressant de souligner que, parmi 33 sérums provenant de malades atteints d'hémopathies malignes, nous avons trouvé 3 cas de phénotype Gm (a- b- x-). Il s'agit d'un cas de thymome malin, d'un cas de plasmocytome, et d'un cas de maladie de Hodgkin, tous à un stade très avancé de leur évolution. Ces sérums ont été examinés par nous, à plusieurs reprises (4 ou 5 fois) et ont toujours donné les mêmes résultats.

La deuxième partie de cette étude a été consacrée au dépistage, dans ces sérums, de substances anti-gamma globuliniques et, en cas de présence de ces substances, à la détermination de leur spécificité anti-Gm.

Sur 1.363 sérums provenant de malades atteints de tumeurs malignes, nous avons trouvé 132 sérums (9,68%) qui ont montré un pouvoir d'agglutiner les globules rouges sensibilisés par des sérums anti-D sélectionnés, dont le facteur Gm était connu; le titre d'agglutination variait de 1/20 à 1/640. (Tableau 2). Le pourcentage est statistiquement différent de celui observé chez les malades non cancéreux.

Tableau 1. *Fréquence des divers phénotypes* Gm *(a), (b), (x), chez les cancéreux*

Phénotypes Gm	Population normale (Moullec et Coll.)	Cancéreux	Malades non cancéreux
a+ b+ x+	84 (11,92%)	73 (11,89%)	15
a+ b− x+	33 (4,68%)	31 (5,05%)	3
a+ b+ x−	211 (29,93%)	179 (29,15%)	26
a+ b− x−	30 (4,25%)	26 (4,24%)	7
a− b+ x−	347 (49,22%)	302 (49,18%)	49
Nombre total examiné	705	611	100

La probabilité que ces deux distributions soient dues au hasard, se situe entre 1% et 1‰. L'étude en fonction de la localisation des tumeurs et en fonction de l'histologie (Tableau 3) n'apporte pas de renseignements complémentaires. Il n'y a pas, en particulier, de différence significative entre les résultats obtenus pour les épithéliomas et les sarcomes ($\chi_2 = 1{,}05$). Parmi 228 sérums de malades non cancéreux, il y avait 8 sérums (3,50%) possédant la substance anti-gamma globuline.

Tableau 2. *Distribution des antiglobulines en fonction de la localisation des tumeurs*

Localisations	Nombre de malades	Nombre des sérums possédant une anti-globuline
Uterus et muqueuses génitales	282	20
O.R.L.	248	28
Sein	239	20
Peau	106	13
Hémopathies	98	6
Tractus digestif	90	13
Glandes endocrines	82	8
Bronches	77	9
Vessie-Rein	48	7
Sarcomes non hémopoïétiques	30	5
Prostates	13	1
Diverses origines	50	4
Total	1363	132 (9,68%)
Malades non cancéreux	228	8 (3,50%)

La spécificité anti-Gm a été établie pour 22 de ces sérums dont 10 anti-Gm (a), 5 anti-Gm (b) et 6 anti-Gm (x). Un seul anti-Gm (b) a été trouvé parmi les sérums de malades non cancéreux.

En comparant le spécificité de ces sérums avec leur groupe sérique, nous avons constaté la présence de l'anti-Gm (a) aussi bien dans le sérum de groupe Gm (a+) (3 sur 10) que dans le sérum Gm (a—). De même, la substance anti-Gm (b) a été trouvée dans les sérums de groupe Gm (b+) (3 sur 5) et Gm (b—). Par contre, l'anti-Gm (x) n'a pu être décelé que dans les sérums dont le phénotype était dépourvu de groupe Gm (x).

Pour répondre à la question de la relation entre les facteurs rhumatoïdes et l'apparition de la substance agglutinante dans les sérum de nos malades, nous avons effectué, sur un certain nombre de sérums, la réaction de Waaler-Rose et le test au Latex-globuline.

En voici les résultats:

— Sur les 132 sérums de cancéreux présentant la substance anti-gamma globuline, le test au Latex a été trouvé positif dans 23 cas (17,4%).

— Sur 105 de ces sérums, la réaction de Waaler-Rose était positive dans 16 sérums (15,2%). Les titres de l'agglutination variaient entre 1/32 et 1/128.

— Parmi les 8 sérums de malades non cancéreux, trois ont montré une réaction de Waaler-Rose et un test au Latex positifs.

— Sur 22 sérums ayant des agglutinines spécifiques anti-Gm (a, b, ou x), la réaction de Waaler-Rose et le test au Latex se révélaient positifs dans 5 sérums, et le titre d'agglutinines anti-Gm était très élevé, variant entre 1/80 et 1/640.

Simultanément avec les sérums possédant le pouvoir agglutinant, nous avons cherché les facteurs rhumatoïdes dans les sérums ne possédant pas la substance anti-gamma globulinique. Sur 500 sérums examinés, la réaction de Waaler-Rose a été positive dans 9 sérums (1,8%) et le test au Latex positif dans 20 de ces

Tableau 3. *Distribution des antiglobulines en fonction de l'histologie des tumeurs*

	Nombre de cas	Antiglobuline		
		Non spécifique	Spécifique	Total
Epithélioma malpighien	730	63 (8,63%)	12 (1,64%)	75 (10,27%)
Epithélioma glandulaire	505	44 (8,71%)	4 (0,79%)	48 (9,50%)
Sarcomes	128	4 (3,20%)	5 (3,92%)	9 (7,12%)
Total	1363	111 (8,14%)	21 (1,54%)	132 (9,68%)
Malades non cancéreux	228	7 (3,07%)	1 (0,43%)	8 (3,50%)

sérums (4%). Il faut souligner que le titre de la réaction de Waaler-Rose dans ce groupe de sérums était très faible, dépassant rarement la dilution 1/32.

Influence des transfusions sur l'apparition des substances anti-gamma globuliniques

Certaines observations ont démontré une influence nette des transfusions sur l'apparition de la substance anti-globulinique (2,25). Nos études concernant les malades transfusés et non transfusés sont présentées dans le Tableau 4. Il y a une différence hautement signicative entre ces deux populations. La probabilité que les deux distributions soient dues au hasard est inférieure à 1°/₀₀ (χ_2=26,4).

nante, et 11 sérums (1,17%) ont une spécificité anti-Gm.

Sur 228 sérums provenant de malades non cancéreux et non transfusés, la substance anti-gamma globulinique a été trouvée présents dans 8 sérums (3,5%). (Il nous faut signaler que quelques malades cancéreux classés dans le groupe "non transfusés" au cours de cette étude, ont subi une intervention chirurgicale, il y a quelques mois ou années, sans qu'il nous ait été possible de vérifier, si à cette époque, ils avaient été transfusés).

Substance agglutinante en fonction de l'age

D'après les résultats publiés récemment, 12,5% de sérums provenant de personnes

Tableau 4. *Distribution des antiglobulines en fonction des transfusions*

Malades cancéreux	Nombre de malades	Les sérums possédant une antiglobuline		
		Non spécifique	Anti-Gm spécifique	Total
Transfusés	421	57 (13,54%)	10 (2,37%)	67 (15,91%)
Non transfusés	942	54 (5,73%)	11 (1,17%)	65 (6,90%)

Sur 421 sérums de malades atteints de tumeurs malignes, examinés après plusieurs transfusions, la substance anti-gamma globulinique a été présente dans 57 sérums (13,54%), et 10 sérums (2,37%) ont eu des agglutinines spécifiques anti-Gm (a, b, ou x). Le nombre de transfusions variait de 3 à 25. Quelques-uns de ces malades ont reçu des transfusions massives au cours d'une intervention chirurgicale. Dans 3 de ces cas, la substance agglutinante, absente avant le transfusion, a été trouvée présente après les transfusions.

Parmi 942 malades cancéreux qui n'ont pas été transfusés, nous en avons trouvé 54 (5,73%) dont les sérums montrent l'aptitude agglutinante, âgées de plus de 70 ans possèdent une aptitude agglutinante anti-gamma globulinique [20]. Le même pourcentage de ces sérums a montré la présence de facteurs rhumatoïdes (Réaction de Waaler-Rose et test au Latex positifs), sans qu'on puisse constater les signes d'une maladie rhumatismale.

Nos résultats, indiquant la présence de la substance anti-gamma globulines dans le sérum de malades atteints de tumeurs malignes,

Tableau 5. *Répartition des anti-globulines en fonction de l'âge*

Age	Nombre de malades	Les sérums possédant une antiglobuline		
		Non spécifique	Anti-Gm spécifique	Total
Moins de 50 ans	355 (27,4%)	19 (5,63%)	0	19 (5,63%)
De 50 a 70 ans	785 (56,90%)	66 (8,40%)	18 (2,29%)	84 (10,69%)
Plus de 70 ans	223 (15,70%)	26 (11,66%)	3 (1,35%)	29 (13,01%)

en fonction de l'âge, figurent dans le Tableau 5.

Sur 1,363 malades cancéreux, 355 (27,4%) étaient âgés de moins de 50 ans, 785 (56,9%) âgés de 50 à 70 ans, et 223 (15,7%) âgés de

plus de 70 ans.

Sur l'ensemble des sérums de ces malades, nous avons trouvé 132 sérums qui ont montré une activité anti-gamma globulinique; 19 sérums (5,63%) proviennent de malades âgés de 50 ans, 84 sérums (10,69%) de malades âgés de 50 à 70 ans, et 29 sérums (13,01%) de malades âgés de plus de 70 ans. La probabilité que cette distribution soit due au hasard est inférieure à 1% ($\chi_2=10,5$).

La spécificité anti-Gm (a, b ou x) a été décelée dans le sérum de 18 malades âgés de 50 à 70 ans et dans le sérum de 3 malades âgés de plus de 70 ans.

DISCUSSION

Ces résultats montrent que la fréquence globale de groupes Gm (a), Gm (b), Gm (x), chez les malades atteints de tumeurs malignes, ne diffère pas de celle constatée chez les sujets normaux et les malades non cancéreux.

En analysant nos résultats du point de vue de la localisation des tumeurs, on note une diminution de la fréquence du facteur Gm (a) dans les cas de sarcomes ou d'hémopathies malignes.

La présence du phénotype pathologique Gm (a- b- x-), et une diminution notable du pourcentage du facteur Gm (a), qui peut aboutir à la disparition complète du facteur Gm (x), a été observée chez les sujets atteints de leucémie lymphoïde et de leucémie aiguë [11–14].

On a aussi constaté des variations phénotypiques de facteur Gm dans certains cas de myélomes, au cours de l'évolution de la maladie [7–11].

On pourrait supposer que la diminution de la fréquence de facteurs Gm est liée à un trouble global des globulines. En effet, chez plusieurs malades cancéreux, nous avons constaté une nette hypo- ou dysgamma globulinémie. Mais cette hypothèse n'explique pas tous les cas observés. Chez certains malades, ni le dosage immunologique de gamma globulines, ni les études électrophorétiques, n'ont montré de troubles des gamma globulines.

L'affaiblissement ou la disparition complète de certains groupes Gm rappellent des modifications d'antigènes érythrocytaires et des anticorps sériques signalés au cours du processus leucémique [21]. Récemment, on a posé la question de savoir si les modifications de ces antigènes Gm, génétiquement contrôlés, ne sont pas dues aux altérations chromosomiques observées dans certains cas de leucoses et de tumeurs malignes [14].

L'apparition de la substance anti-globulinique n'a pas trouvé, à ce jour, d'explication satisfaisante.

Trois éléments doivent être pris en considération:

(1) Processus immunologique au cours de la maladie tumorale;

(2) L'âge des malades étudiés;

(3) Les transfusions.

Peut-on parler d'une auto- ou isoimmunisation?

L'hypothèse d'une autoimmunisation par des globulines changées au cours d'un processus immunologique a été suggérée [22, 23]. Les sérums SNAGG, provenant de sujets sains, (donneurs de sang) témoignent contre cette hypothèse. La production de RAGG chez les malades atteints de PCE semble plaider en faveur d'une autoimmunisation.

Un mécanisme semblable pourrait expliquer l'apparition de l'anti-gamma globuline dans les sérums de cancéreux.

La notion d'autoimmunisation, dans la maladie néoplasique, semble gagner du terrain durant ces dernières années. Plusieurs études ont démontré la présence d'auto-anticorps antitumoraux dans les sérums de certains malades cancéreux [24]. On peut se demander si l'auto-antigène libéré par le tissu cancéreux, responsable de la production des auto-anticorps antitumoraux, pourrait stimuler, au cours du processus néoplasique, des réactions en chaîne, comportant l'apparition de la substance anti-globulinique.

Les examens systématiques chez des sujets âgés de 70 ans ont démontré l'existence des dysglobulinémies sériques, appartenant aux mêmes types immunochimiques que les protéines myélomateuses ou macroglobulinémiques. Ces sujets ne présentaient pas de signes pathologiques de ces affections [25]. D'autre part, l'augmentation de fréquence de la substance agglutinante (12,5%) et des facteurs rhumatoïdes, dans le sérum des vieillards chez qui l'on n'a pas trouvé de signes de maladie rhumatismale, a été constatée [19–26]. Cette fréquence élevée de l'activité agglutinante de ces sérums est à rapprocher des perturbations protéiques notées chez les sujets âgés.

L'analyse de nos résultats, en fonction de l'âge (Tableau 5), montre que la fréquence de la substance anti-gamma globulines augmente régulièrement avec l'âge. Elle est de 5,63% dans les sérums de malades dont l'âge ne dépasse pas 50 ans, de 8,4% lorsque l'âge est entre 50 et 70 ans, et de 11,66% chez les malades âgés de plus de 70 ans.

Chez les malades atteints de PCE, les

auteurs proposent de considérer l'apparition et la persistance des anti-Gm comme le signe d'une évolution sévère de mauvais pronostic. Il nous paraît difficile d'élargir ces conclusions aux malades atteints de néoplasies malignes.

L'élément qui peut jouer un rôle important dans l'apparition de la substance anti-globulinique chez les malades cancéreux, est l'isoimmunisation par les transfusions.

Certaines observations et expérimentations semblent le confirmer. On a pu montrer que la transfusion de plasma Gm (a+) à un volontaire Gm (a−), contenant un titre élevé d'anti-Gm (a) (IgG), a provoqué une réaction post-transfusionnelle [9].

Chez les enfants atteints de thalassémie et polytransfusés, on a constaté la présence d'anti-Gm [2, 31]. L'incompatibilité foeto-maternelle de groupes Gm (a), pendant la grossesse, a provoqué, dans certains cas, l'apparition d'anti-Gm (a) [28–30]. Le titre du SNAGG anti-Inv (b) a été trouvé nettement diminué après une transfusion de sang total Inv (b+) [25].

Dans cette étude sur 421 sérums de malades cancéreux ayant reçu des transfusions, nous avons trouvé 67 sérums (15,9%) avec la substance anti-globulinique. Par contre, parmi 942 malades qui n'ont pas été transfusés, 65 seulement (6,9%) ont présenté l'activité agglutinante. Parmi nos 228 sérums de malades non cancéreux, 8 (3,5%) ont montré le pouvoir d'agglutiner les globules rouges sensibilisés.

En conclusion, nous avons constaté dans environ 10% des sérums provenant de malades atteints de tumeurs malignes, des anti-gamma globulines à un titre assez élevé, Dans certains cas, ces antiglobulines sont dirigées contre le propre facteur Gm du malade. L'apparition d'une autoimmunisation par les gamma globulines modifiées au cours du processus tumoral paraît donc probable. D'autre part, nous avons confirmé, chez les personnes âgées et polytransfusées, l'accroissement des substances anti-globuliniques, ce qui montre avec quelle prudence il faut interpréter, chez les malades cancéreux, la découverte d'une substance dysglobulinique.

Note:

Suivant la nomenclature des facteurs au locus Gm proposée par O.M.S. [*Bull. Wld Hlth* **33,** 721 (1965)] les facteurs Gm (a), Gm (x) et Gm (b) sont désignés respectivement par Gm (1), Gm (2) et Gm (5).

RÉSUMÉ

On a recherché la présence des substances anti-globulines humaines en fonction de la répartition des antigènes sériques Gm(a), Gm (b) et Gm (x) dans le sérum de 1.363 *cancéreux et de* 228 *malades non cancéreux. On a trouvé des substances anti-globulines humaines chez* 9,68% *des cancéreux et chez* 3,5% *des malades non cancéreux. Ces anti-globulines sont plus fréquentes chez les malades transfusés* (13,54) *que chez les malades non transfusés* (5,73). *La fréquence s'accroît aussi en fonction de l'âge* (5,63% *avant* 50 *ans,* 8,4% *entre* 40 *et* 70 *ans,* 11,66% *après* 70 *ans*). *On peut déceler une spécificité anti-Gm dans ces substances anti-globuliniques* (10 *cas sur* 57 *chez les transfusés,* 11 *cas sur* 54 *chez les non transfusés*). *Cette spécificité anti-Gm s'observe surtout entre* 50 *et* 70 *ans* (18 *cas sur* 66). *Elle est plus rare après* 70 *ans* (3 *cas sur* 26). *Elle n'a pas été décelée au-dessous de 50 ans. L'origine immunitaire des ces substances dans les sérums de cancéreux est discutée.*

SUMMARY

Human anti-globulins in relation to the frequency of Gm (a), (b) and (x) groups were investigated in the serum of 1363 *patients affected with malignant tumours, and* 228 *non-cancerous patients. They were found in* 9·68% *of patients with maligant tumours, and in* 3·5% *of non-cancerous patients. The incidence of anti-globulin agglutinators was higher among patients who underwent numerous transfusions* (13·54%) *than among non-transfused patients* (5·73%). *The frequency also increased in proportion to age* (5·63%) *before* 50 *years,* 8·4% *from* 50 *to* 70 *years and* 11·66% *after* 70 *years*). *Specific anti-Gm (a), (b) and (x) agglutinators were found in* 21 *sera* (10 *cases out of* 57 *transfused patients and* 11 *cases out of* 54 *non-transfused patients*). *They are most frequent among patients aged from* 50 *to* 70 *years, and less frequent after* 70 *years; they were not observed in patients under* 50 *years.*

The appearance of the antiglobulin factors as a consequence of an immunological process in cancerous patients is discussed by the authors.

ZUSAMMENFASSUNG

Die Anwesenheit von Antiglobulinantikörpern im Verhältnis zu der Häufigkeitsverteilung von Gm (a), (b) und (x) Faktoren wurde im Serum von 1363 *Krebskranken und* 228 *mit sonstigen Krankheiten behafteten Patienten untersucht. Wir fanden sie in* 9,68% *der Krebskranken und in* 3,5% *der sonstigen Kranken. Das Auftreten der Antikörper gegen Gammaglobulin war häufiger bei polytransfundierten Kranken* (13,54%) *als bei nichttransfundierten Kranken* (5,73%). *Die Häufigkeit nahm ebenfalls proportionnell mit dem Alter zu* (5,63% *unter* 50 *Jahren,* 8,40% *zwischen* 50 *und* 70 *Jahren,* 11,66% *nach* 70 *Jahren). In* 21 *Seren wurden spezifische Anti-Gm (a), (b) und (x) Antikörper gefunden.* (10 *Fälle bei* 57 *transfundierten und* 11 *Fälle bei nichttransfundierten Kranken). Diese Antikörper treten am häufigsten unter Kranken im Alter zwischen* 50 *und* 70 *Jahren auf; nach* 70 *Jahren kommen sie nur selten vor. Unter* 50 *Jahren wurden sie in unserem Untersuchungsmaterial nicht gefunden.*

Das Auftreten der Antiglobulinantikörper im Zusammenhang mit einem immunologischen Mechanismus bei Krebskranken wird von den Autoren erörtert.

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Alcool et Cancer

Etude de Pathologie Géographique Portant sur 19 Pays

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L'HYPOTHÈSE d'une association entre la consommation d'alcool et certains cancers a été soulevée à diverses reprises. Elle repose sur des arguments de deux ordres:

Arguments indirects

La mortalité très élevée par cancer de certaines localisations chez les sujets dont la profession les amène à consommer beaucoup d'alcool: barman, aubergiste, représentant [1, 2].

La corrélation positive, observée en France par Sully Ledermann, entre les taux, par département, de mortalité par alcoolisme et par cancer de toutes localisations [3, 4].

La constatation, au cours d'une enquête de morbidité par cancer, réalisée dans certains départements français, de l'incidence particulière du cancer de l'oesophage dans le Calvados et la Manche, régions à forte consommation d'alcool [5].

Arguments directs

Ils proviennent d'enquêtes rétrospectives:

On a comparé la consommation d'alcool de cancéreux avec celle de témoins appariés, et noté une association positive pour les cancers de la langue, de l'hypopharynx, du larynx, de l'oesophage, et sans doute de la cavité buccale et de l'oropharynx [6–12].

On a également comparé entre elles les diverses localisations de cancer [13, 14] et remarqué une consommation particulièrement élevée pour les localisations aéro-digestives supérieures.

L'ensemble de ces arguments, bien qu'ils suscitent des réserves, convergent vers une même présomption.

Notre propos, dans cette étude de pathologie géographique, a été de chercher de nouveaux arguments en faveur d'une association possible alcool-cancer.

MATERIEL ET METHODE

Nous avons retenu 19 pays pour lesquels nous disposions de renseignements suffisants concernant l'alcoolisme et le cancer; ce sont: l'Angleterre, l'Australie, l'Autriche, la Belgique, le Canada, le Danemark, l'Ecosse, les Etats-Unis, la Finlande, la France, l'Irlande, l'Italie, le Japon, la Norvège, les Pays-Bas, le Portugal, la République Fédérale Allemande, la Suède et la Suisse.

Nous avons estimé le *degré d'alcoolisme:*

(a) indirectement par le dénombrement des décès par cirrhose [15],

(b) directement à partir de la consommation de bière et de vin [16] connue dans 13 des 19 pays étudiés, à l'exclusion de l'Angleterre, l'Australie, le Canada, l'Ecosse, les Etats-Unis, le Japon.

Pour les *cancers*, nous nous sommes reportés aux dénombrements publiés dans les annuaires statistiques et épidémiologiques de l'O.M.S., dans lesquels figurent, pour chaque pays, par sexe et par tranche d'âge de 5 ans, les nombres absolus de décès, selon les rubriques de la liste intermédiaire (liste A) de la classification

*Avec la collaboration technique de M. H. TRAN.

internationale des maladies, traumatismes et causes de décès.

Notre étude porte sur les *hommes de plus de* 45 *ans*, les décès par cirrhose et par cancer étant rares avant cet âge et de *moins de* 65 *ans* de façon à réduire le pourcentage de décès de causes indéterminées, et elle concerne les *années* 1958–1959 *et* 1960.

Nous avons calculé:

(a) à partir des *nombres absolus de décès* par cirrhose et pour chaque cancer: des *taux bruts* de mortalité en effectuant le quotient du nombre de décès signalés en 1958–1959 et 1960, par le nombre d'habitants recensés en 1959, année médiane de cette étude, multiplié par trois. Nous avons corrigé ces taux bruts de l'influence:

des décès de "causes indéterminées", en faisant l'hypothèse que la proportion de décès par cancer, dissimulée sous la rubrique "décès de causes indéterminées", est la même que la proportion des décès par cancers rapportés à l'ensemble des décès de causes connues: (si x=taux brut de décès par cancer, y=pourcentage de décès de causes indéterminées, rapporté à l'ensemble des décès, le taux corrigé $x'=x/1-y$).

de la mortalité générale (si x'=taux corrigé des "causes indéterminées, z=taux de mortalité générale, le taux corrigé $x''=x'/z$).

Le taux x'' exprime le pourcentage de décès attribuables à la cirrhose et au cancer dans la mortalité générale, après correction des causes indéterminées.

(b) à partir des *consommations annuelles* de bière et de vin par habitant: une consommation en "degré alcoolique" en attribuant au vin un degré alcoolique moyen de 10° et à la bière un degré alcoolique moyen de 5°.

Nous avons classé les pays selon leur consommation de vin, de vin et de bière, et attribué un "rang" à chacun d'eux.

RESULTATS

A. *Corrélations entre la mortalité par cirrhose et la mortalité par diverses localisations de cancer* (Tableau 1).

Avec les taux bruts (colonne 3), on note une corrélation significative positive pour les cancers de la *cavité buccale et du pharynx* (r=0,73), de *l'oesophage* (r=0,67), du *larynx* (r=0,80), des *os et du tissu conjonctif* (r=0,65).

Avec les taux corrigés (colonne 4), les résultats sont pratiquement les mêmes.

La situation de certains pays, en particulier la France, risquant de peser trop sur la valeur des coefficients de corrélation calculés à partir des taux, nous avons utilisé les coefficients de corrélation des rangs (Tableau 2).

Leur valeur est atténuée mais ils restent significatifs, sauf ceux de la cavité buccale et du pharynx.

Tableau 1. *Corrélation entre les taux de mortalité par cirrhose et par cancer*

Désignation selon la classification internationale des causes de décès (liste intermédiaire)	Localisation	Corrélation avec la mortalité par cirrhose	Corrélation avec la mortalité par cirrhose, en tenant compte des décès de cause indéterminée et de la mortalité générale
A 44	Cavité buccale et pharynx	0,73+++	0,74+++
A 45	Oesophage	0,67++	0,70+++
A 46	Estomac	0,10	0,06
A 47	Intestin	−0,20	−0,28
A 48	Rectum	−0,21	−0,26
A 49	Larynx	0,80+++	0,82+++
A 50	Trachée, poumon	−0,34	−0,46+
A 54	Prostate	0,07	−0,06
A 55	Peau	−0,21	−0,25
A 56	Os et tissu conjonctif	0,65+++	0,62++
A 58	Leucémies	−0,06	−0,17

Seuil de signification: $+p\leqslant 0{,}05$ $++p\leqslant 0{,}01$ $+++p\leqslant 0{,}001$

Tableau 2. Corrélation des rangs entre les taux de mortalité par cirrhose et par cancer

Désignation selon la classification internationale des causes de décès (liste intermédiaire)	Localisation	Corrélation des rangs avec la mortalité par cirrhose
A 44	Cavité buccale et pharynx	0,45
A 45	Oesophage	0,49+
A 49	Larynx	0,68++
A 56	Os et tissu conjonctif	0,55+

Seuil de signification: + $p \leqslant 0{,}05$ ++ $p \leqslant 0{,}01$ +++ $p \leqslant 0{,}001$

B. *Corrélations entre les consommations annuelles et les taux de mortalité par diverses localisations de cancer* (Tableau 3)

Pour la consommation de vin, une corrélation significative positive est trouvée avec les cancers de la *cavité buccale et du pharynx* ($r=0{,}65$), du *larynx* ($r=0{,}78$), et des *os et du tissu conjonctif.* ($r=0{,}61$).

Pour la consommation "vin+bière", la corrélation avec le *cancer du larynx* ($r=0{,}80$) subsiste, elle n'est par contre plus significative pour les cancers de la cavité buccale et du pharynx, de l'oesophage ainsi que des os et du tissu conjonctif.

Tableau 3. Corrélation des rangs entre la consommation annuelle de vin, vin et bière et la mortalité par cancer

Désignation selon la classification internationale des causes de décès (liste intermédiaire)	Localisation	Corrélation des rangs avec la consommation de vin	Corrélation des rangs avec la consommation vin+bière
A 44	Cavité buccale et pharynx	0,53	0,52
A 45	Oesophage	0,65+	0,50
A 46	Estomac	0,27	−0,30
A 47	Intestin	−0,18	−0,10
A 48	Rectum	−0,27	−0,12
A 49	Larynx	0,78++	0,80++
A 50	Trachée, poumon	−0,35	−0,36
A 54	Prostate	−0,04	−0,24
A 55	Peau	−0,35	−0,56
A 56	Os et tissu conjonctif	0,61+	0,54
A 58	Leucémies	−0.08	−0,34

Seuil de signification: + $p \leqslant 0{,}05$ ++ $p \leqslant 0{,}01$ +++ $p \leqslant 0{,}001$

DISCUSSION

(a) Les documents de mortalité utilisés sont basés sur les certificats de décès dont on connaît l'imprécision; nous avons essayé d'en tenir compte, dans une certaine mesure, en nous situant dans une tranche d'âge (45–64 ans) dans laquelle la proportion de causes indéterminées est la moins élevée et en corrigeant les taux bruts.

Par ailleurs, la nomenclature des causes de décès selon la liste intermédiaire (liste A) donne une classification assez grossière, notamment en groupant les cancers de la cavité buccale et du pharynx et en ne distinguant pas les localisations oropharyngées et hypopharyngées.

(b) La mesure du degré d'alcoolisme par le nombre de décès par "cirrhose" ne constitue qu'une approche; en effet, d'une part, la cirrhose n'est qu'une manifestation de l'alcoolisme, d'autre part, elle n'est pas toujours d'origine alcoolique et si l'on peut admettre qu'en France 80% des cirrhoses sont d'origine alcoolique [17], dans d'autres pays, cette proportion est beaucoup plus faible.

Toutefois, la connaissance de la consommation de vin et de bière dans 13 des 19 pays étudiés nous a permis de vérifier qu'il existait

une corrélation significative positive entre la mortalité par cirrhose et la consommation de vin ($r=0,93$) ou la consommation de "vin+bière" ($r=0,81$). En outre, nous avons retrouvé les mêmes corrélations entre la consommation de vin et les cancers. Cette constatation atténue sensiblement nos réserves.

(c) Enfin, il demeure que nous avons mesuré la liaison alcool-cancer par le coefficient de corrélation, calculé à partir de deux variables se rapportant à des groupes distincts de sujets. Nous savons que la mise en évidence de liaisons au niveau de groupes ne prouve pas qu'elles existent au niveau des sujets constituant ces groupes.

(d) En ce qui concerne les résultats, certains vont dans un sens "attendu": les corrélations significatives positives avec le cancer de la *cavité buccale et du pharynx*, de *l'oesophage*, du *larynx*. Nous les observons aussi bien avec la mortalité par cirrhose qu'avec la consommation de vin et de "vin+bière". C'est là un argument de plus en faveur d'une association entre l'alcoolisme et ces cancers. Il faut toutefois noter que cette étude ne nous permet pas de distinguer le rôle étiologique du rôle pronostique éventuel de l'alcool; les résultats observés, du fait qu'ils sont basés sur la mortalité, s'expliqueraient aussi bien si l'alcoolisme hâtait l'évolution de cancers pour lesquels il n'aurait aucun rôle étiologique.

Un résultat par contre est inattendu: la corrélation avec le cancer des os et du tissu conjonctif.

On peut se demander tout d'abord ce que recouvre cette rubrique.

Le "cancer des os et du tissu conjonctif" (A 56 dans la liste intermédiaire de la classification internationale des maladies et causes de décès) désigne deux sortes de localisations: les tumeurs de l'os (196 de la liste détaillée de la classification internationale des maladies et causes de décès) et les tumeurs du tissu conjonctif (197).

A partir de documents transmis par l'O.M.S.*, nous avons pu dissocier les deux rubriques dans 11 des 19 pays, et calculer le coefficient de corrélation entre la mortalité par cirrhose et d'une part le cancer des os ($r=0,69$) d'autre part le cancer du tissu conjonctif ($r=0,18$ non significatif). La corrélation ne subsiste qu'avec le cancer des os.

En second lieu, on peut se demander si la rubrique cancer des os ne recouvre pas un certain nombre de métastases secondaires à des localisations primitives qui seraient elles-mêmes responsables de l'association avec l'alcool.

Nous avons pu vérifier, dans des documents issus d'une part de l'Enquête Permanente Cancer*, d'autre part de "U.S. End Results Group"†, que les tumeurs enregistrées comme primitives recevaient une confirmation histologique dans respectivement 77% des cas et 93% des cas.

Bien qu'il s'agisse d'observations hospitalières, ces pourcentages nous paraissent suffisamment élevés pour indiquer que, dans l'ensemble des cas, la proportion des localisations osseuses primitives est relativement importante.

Par ailleurs, on peut souligner que les cancers qui donnent le plus souvent des métastases osseuses chez l'homme (prostate, poumon, thyroïde, rein . . .) concernent des localisations au niveau desquelles l'alcool ne paraît devoir jouer aucun rôle.

On peut ensuite se demander quelle est la répartition précise des cancers primitifs de l'os sur le squelette.

Nous avons, à cet effet, étudié des documents provenant de 5 pays: Danemark, Ecosse, France, Italie, Suède (Tableau 4.)

Tableau 4. *Répartition des localisations osseuses*

	Crâne max. infér.	Vertèbres	Membres	Total
<45 ans	13% (62)	13% (59)	74% (337)	458
⩾45 ans	32% (685)	23% (492)	45% (967)	2144

Si, avant 45 ans, comme cela est classique, c'est la localisation aux membres qui est la plus fréquente; par contre, après 45 ans, c'est-à-dire la zone d'âge qui nous intéresse, cette proportion est nettement plus faible. On voit qu'environ 1/3 des cancers sont localisés aux os de crâne et du maxillaire inférieur. On peut penser que cette rubrique recouvre en fait des localisations de la sphère oto-rhino-laryngologique dont on connaît la relation avec l'alcool.

*Nous remercions vivement le Docteur Logan et le Docteur N. Grais de nous avoir communiqué ces documents.

*Enquête conduite par la Section Cancer de l'Institut National de la Santé et de la Recherche Médicale grâce à la coopération de 21 centres anticancéreux français.

†Nous remercions vivement le Docteur S. J. Cutler de nous avoir transmis les données de U.S. End Results Group.

Nous avons cherché des arguments susceptibles d'étayer cette hypothèse. Dans une étude précédente (18, 19), on a constaté que le sex-ratio pouvait être considéré comme une mesure de la liaison de certains cancers avec des facteurs exogènes tels le tabac ou l'alcool, la prédominance masculine étant d'autant plus marquée que la liaison est plus forte. Nous avons donc calculé le sex-ratio pour les différents sièges du cancer des os (Tableau 5).

Tableau 5. *Sex-ratio selon les différents sièges, après* 45 *ans*

Crâne max. infér.	Vertèbres	Membres
2,06	1,31	1,15

On voit que le sex-ratio pour les localisations "crâne et maxillaire inférieur" est significativement plus élevé qu'au niveau des autres sièges.

Cette prédominance masculine cadre assez bien avec l'hypothèse d'une association de ces localisations avec le facteur alcool.

Il reste toutefois à expliquer comment la corrélation entre la consommation d'alcool et la mortalité par cancer des os de toutes localisations, peut être aussi importante si elle provient en fait uniquement des localisations "crâne-maxillaire inférieur" qui constituent 1/3 des cas. Il faudrait admettre que cette zone présente une corrélation avec l'alcoolisme très élevée, et/ou une mortalité particulièrement forte.

Tant que ces points n'ont pas été éclaircis, on ne peut exclure l'hypothèse d'une corrélation entre l'alcoolisme et le cancer des os de toutes localisations; cette dernière éventualité amènerait à chercher une explication d'ordre biologique.

Nous pensons que l'étude, dans son ensemble, mérite d'être poursuivie.

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En collaboration avec la Section "Cancer" de l'Institut National de la Santé et de la Recherche Médicale.

RÈSUMÈ

Il s'agit d'une étude de pathologie géographique dans laquelle on a étudié, au niveau de 19 *pays, la corrélation entre la mortalité par cancer et l'alcoolisme, celui-ci étant mesuré, soit par la mortalité par cirrhose, soit par la consommation d'alcool.*

Cette étude confirme l'existence d'une relation entre les cancers des voies aéro-digestives supérieures et de l'oesophage, d'une part, et l'alcoolisme, d'autre part. Il n'est cependant pas possible de préciser la part qui revient à un facteur étiologique déclenchant et celle qui revient à un facteur pronostique aggravant.

Elle met ensuite en évidence une corrélation positive inattendue pour le cancer des os. Une explication possible serait que cette rubrique recouvre certaines localisations aéro-digestives supérieures en relation avec l'alcoolisme, mais ce n'est qu'une hypothèse qui appelle confirmation.

SUMMARY

A study of geographical pathology has been carried out in 19 *countries in which the correlation between cancer mortality and alcoholism was investigated, the latter being measured either by cirrhosis mortality or alcohol ingestion.*

This study confirms that a relation exists between cancer of the upper aero-digestive region, and of the oesophagous, and alcoholism. However, it is not possible to specify whether the correlation is accounted for by an etiological factor or whether it is due to a factor which carries a bad prognosis.

In addition, an unexpected and positive correlation with bone cancer was found. This could conceivably be due to bone metastases associated with cancer of the upper digestive tract caused by alcohol. There is, however, no data available to support this hypothesis.

ZUSAMMENFASSUNG

In 19 Ländern wurde ein geographisch-pathologisches Studium, welches das Verhältnis zwischen der Krebssterblichkeit und dem Alkoholismus erfassen sollte, durchgeführt. Der Alkoholismus wurde entweder aufgrund der Zirrhosensterblichkeit erfasst, oder an dem Alkoholverbrauch gemessen.

Es wurde dadurch bestätigt, dass es zwischen den Karzinomen der oberen Atem- und Verdauungswege und der Speiseröhre einerseits und dem Alkoholismus andererseits eine Korrelation besteht. Es ist jedoch unmöglich zu bestimmen inwieweit es sich um einen ätiologischen Faktor handeln könnte, der die Erkrankung selbst hervorriefe, oder um einen solchen, der nur die Prognose verschlechterte. Es wurde weiter, unerwartet, eine positive Korrelation zum Knochenkrebs gefunden. Dies kann möglicherweise durch die Knochenmetastasen der Karzinome der oberen Atem- und Verdauungswege, die wiederum mit dem Alkoholismus in Wechselbeziehung stehen sollten, erklärt werden. Es handelt sich hier nur um eine Vermutung, die noch bestätigt werden muss.

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Testosterone Levels in the Plasma of Normal Women and Patients with Benign Breast Disease or with Breast Cancer

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INTRODUCTION

THERE is a close relationship between the amounts of androgen metabolites in the urine and the clinical course of breast cancer. The majority of patients with widespread metastases who excrete subnormal amounts of androsterone and aetiocholanolone do not respond to adrenalectomy or hypophysectomy [1] or to pituitary destruction by yttrium-90 [2]. A similar abnormality in steroid excretion is found in about half the patients with the early disease (before metastatic spread is found) and these patients also have a poor prognosis [3, 4].

The urinary androgen metabolites are derived from at least four precursors (dehydroepiandrosterone and its sulphate, androstenedione, and testosterone) which means that deductions about the concentration of precursors in the blood cannot be made from measurements of urinary metabolites [5]. In an attempt to investigate the physiological meaning of the correlation between urinary steroid levels and the clinical course of breast cancer direct measurements of plasma 17-oxosteroids and 17-hydroxycorticosteroids have been made [6] and preliminary investigations of secretion rates have been carried out [7].

This paper is concerned with the levels of free testosterone in the plasma of normal women and patients with benign breast disease or early breast cancer.

METHODS

(a) *Subjects*

Blood (20 ml) was taken into heparinised syringes from the antecubital vein between 2 p.m. and 4 p.m. for normal women and between 10 a.m. and 11 a.m. for patients with breast disease. The time difference is unlikely to be important since there is no diurnal variation in plasma testosterone levels [8]. The plasma prepared from these samples was stored at —20°. Blood samples from premenopausal women were taken at random during the menstrual cycle. Minor variations in plasma testosterone levels have been reported during the cycle [9] but this variation would be unlikely to alter any of the conclusions in the present study since the proportion of premenopausal women in each of the three groups of subjects was approximately equal.

The patients with benign breast disease consisted of fourteen women admitted to hospital to await diagnosis and subsequent surgery. Their ages ranged from 24 to 59 years (mean 43·4). The patients with breast cancer were twenty-four women, in hospital awaiting radical mastectomy. The ages of these patients ranged from 25 to 84 years (mean 49·7). All diagnoses were confirmed eventually by histological examination. The normal women were nineteen volunteers, apparently healthy, who were housewives living in the North London

area. Their ages ranged from 25 to 76 years (mean 51·9).

(b) *Chemical*

Plasma testosterone was estimated by the method of Burger, Kent and Kellie [10]. Since there was the possibility of contamination of the testosterone fraction by epitestosterone [11] an additional purification step was introduced into the method. This was a preliminary purification of the plasma testosterone extract by paper chromatography (2: 2: 4 trimethylpentane: toluene/methanol: water. 450: 50/400: 100).

(c) *Precision*

Six estimations were carried out on pooled plasma from women. The mean value was 0·069 μg/100 ml plasma with a standard deviation of 0·026 μg/100 ml. Similar experiments on plasma from males gave a mean value of 1·038 μg/100 ml with a standard deviation of 0·071 μg/100 ml. Errors of this order have been reported previously [12] and are due mainly to the relatively large amounts of marker ^{14}C-testosterone added to the plasma. Since the mean level of testosterone found in this study was 0·12 μg/100 ml the coefficient of variation would be of the order of ±20%.

(d) *Specificity*

The ratio of tritium to ^{14}C in the last four chromatographic steps of the analytical method are shown in Fig. 1. The ratios are constant in the last two steps which implies that the testosterone acetate is radiochemically pure.

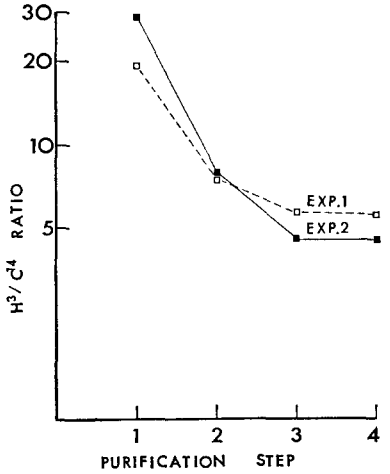

Fig. 1. Ratio of tritium to carbon-14 during purification of doubly labelled testosterone acetate.

RESULTS

The distribution of plasma testosterone levels with age are shown in Fig. 2 for normal women and for patients with benign breast disease or

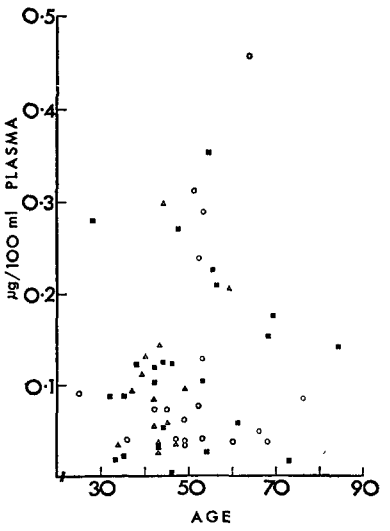

Fig. 2. Plasma testosterone levels and age in normal women, patients with benign breast disease, and patients with early breast cancer.
○ = normal women
△ = women with benign breast disease
■ = women with early breast cancer

breast cancer. There is no correlation with age in any of these groups of patients.

The individual values for each group are shown in Fig. 3: the mean values are almost

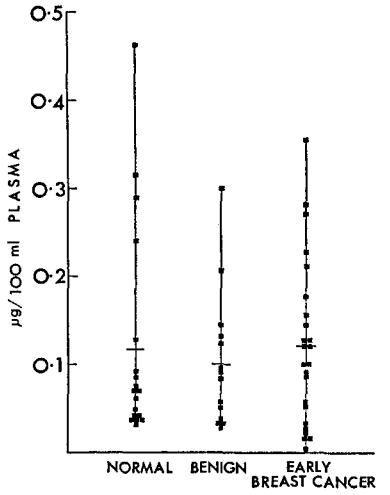

Fig. 3. Comparison of plasma testosterone levels in normal women, patients with benign breast disease, and patients with early breast cancer.

identical. The mean value for normal women is 0·12±0·12 μg/100 ml (standard deviation). These values are in agreement with others in the literature [8, 12, 13]. The mean value for the breast cancer cases is 0·12±0·09 μg/100 ml and is 0·10±0·08 μg/100 ml for those with benign breast disease. There was no correlation between the plasma testosterone concentration and the amounts of androsterone or actiocholanolone in the urine (see also [8, 9]).

DISCUSSION

It has been assumed that the subnormal excretion of urinary androgen metabolites by patients with breast cancer who have a poor prognosis after mastectomy or after endocrine ablation was a reflection of a deficient production of androgens [14, 15]. The present results showing normal plasma testosterone levels in both benign and maligant breast disease indicate that this interpretation of the results of assays of urinary androgen metabolites must be regarded with caution.

The abnormally low urinary excretion, found in some patients, of androsterone and aetiocholanolone is probably related to a deficiency in the secretion of dehydroepiandrosterone and its sulphate. This would be expected since the major portion of the urinary 11-deoxy-17-oxosteroids are derived from these two precursors [5]. Furthermore, a good correlation between the levels of plasma 17-oxosteroids (mainly the sulphates of dehydroepiandrosterone and androsterone) and those of the urinary 11-deoxy-17-oxosteroids has been reported [6].

The measurement of testosterone in plasma has attracted much attention since it was supposed that this compound was the major androgen in women [5]. It has recently been shown [16, 17] that androstenedione is present in much higher concentrations than testosterone in the peripheral blood of normal women and that previous estimates of the amounts of testosterone secreted are probably over-estimates [5].

Before any statement can be made concerning androgenic status in women with breast cancer it would be desirable to obtain simultaneous measurements of both androstenedione and testosterone (and of the rates of their interconversion) in these patients. A more sensitive and precise method than that used in this study such as that employing ^{35}S-labelled thiosemicarbazide [18] would be essential in such an investigation. A second essential factor is the elucidation of the physiological roles of dehydroepiandrosterone and its sulphate in women. It seems unlikely that rapid progress can be made in the investigation of endocrinological aspects of human breast cancer until more is known about the androgenic stimulus in normal women.

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RÉSUMÉ

Chez les patientes atteintes de cancer du sein, il existe une corrélation entre le pronostic de l'affection et les taux urinaires des métabolites des androgènes. Afin de voir si ces données peuvent être étendues aux taux des androgènes dans la circulation périphérique, la testostérone a été dosée dans le plasma de trois groupes de sujets: des femmes normales, des patientes atteintes d'une affection bénigne du sein et des patientes atteintes de cancer du sein à un stade précoce. Aucune différence n'a été observée. Les valeurs moyennes (et la déviation standard) du taux de la testostérone plasmatique furent respectivement dans les trois groupes: $0 \cdot 12 \pm 0{,}12$ *µg/100 ml;* $0{,}10 \pm 0{,}08$ *µg/100 ml et* $0{,}12 \pm 0{,}09$ *µg/100 ml. On ne trouve aucune corrélation entre ces concentrations de testostérone et l'excrétion urinaire des métabolites des androgènes.*

SUMMARY

The prognosis of patients suffering from breast cancer is related to the urinary levels of androgen metabolites. In an attempt to extend this finding, in terms of the levels of androgen in the peripheral circulation, plasma testosterone levels have been determined in three groups of women: normal, patients with benign breast disease, and patients suffering from early breast cancer. No differences in plasma testosterone levels were found between any of the groups, the values for them being $0 \cdot 12 \pm 0 \cdot 12$ *µg/100 ml (standard deviation),* $0 \cdot 10 \pm 0 \cdot 08$ *µg/100 ml and* $0 \cdot 12 \pm 0 \cdot 09$ *µg/100 ml, respectively. No correlation was found to exist between these testosterone levels and the urinary excretion of androgen metabolites.*

ZUSAMMENFASSUNG

Bei den von einem Brustkrebs befallenen Patientinnen besteht ein bestimmtes Verhältnis zwichen der Krankheitsprognose und der Konzentration der im Harn ausgeschiedenen Androgenen Stoffwechselprodukte. Um zu bestimmen, ob diese Angaben auch bezüglich des Androgenen-Spiegels in dem zirkulierenden Plasma gültig sind, hat man den Plasma-Testosteronspiegel bei 3 Gruppen von Patientinnen bestimmt: bei normalen Frauen, bei

solchen, von einer gutartigen Brusterkrankung befallen, und bei Frauen an einem im Frühstadium Brustkrebs leidenden. Man konnte keinen Unterschied bezüglich des Plasma-Testosteronspiegels dieser 3 Gruppen feststellen. Die gefundenen Werte (mit der Standardabweichung) waren wiefolgt: 0,12±0,12 µg/100 *ml;* 0,10±0,08 µg/100 *ml und* 0,12±0,09 µg/100 *ml. Es besteht kein Zusammenhang zwischen diesen Plasma-Testosteronwerte und der Konzentration der mit dem Harn ausgeschiedenen Androgenen Stoffwechselprodukte.*

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Cell Proliferation in Burkitt Tumours

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HISTOLOGICALLY, Burkitt tumour is composed of uniform primitive lymphoid cells interspersed with large, clear or vaculoated non-neoplastic histiocytes. In Giemsa stained imprint or smear preparations of the tumour the lymphoid cells have a constant and characteristic appearance that permits the ready identification of Burkitt tumour and its differentiation from other types of lymphoma or leukaemia [1]. These cells can also be recognised and differentiated from the cells of other types of lymphoma by their behaviour and morphology in tissue culture [2]. Wright and McAlpine [3] have recently drawn attention to the high cytoplasmic RNA content of the Burkitt tumour cells, as demonstrated by pyronine and acridine orange staining. They have suggested that this may be associated with high rates of proliferative activity. The kinetics of the cell proliferation of Burkitt tumour cells grown in tissue culture have indicated that they have a fairly rapid cell cycle time, but that cell death is frequent [4, 5]. Furthermore, it has been observed during the initial months in culture that the chromosome number of the cells was normal, being either diploid or pseudo-diploid [5–7]. However, these studies on the cells *in vitro* give no indication as to the actual rates of proliferation of the tumour cells *in vivo*.

Chromosome studies on fresh biopsy specimens of Burkitt tumour have indicated that the chromosome number is normal or varies only slightly and that marked aneuploidy is not a feature of these tumours despite their rapid growth rate [8]. This stability of the chromosome number of the tumour cells suggested that useful information about the proliferation of these cells *in vivo* could be obtained from a study of the distribution of cell population in the various compartments of interphase activity. A population of interphase cells can be subdivided according to the DNA content of the nuclei and whether evidence of DNA synthesis in the nucleus is present. Following mitosis the interphase nucleus has a diploid DNA content (2c) and for a variable length of time no DNA synthesis occurs; the cell is then said to be in the G_1 compartment of interphase. This is followed by a period of DNA synthesis in which the DNA content of the nucleus doubles from the 2c to the 4c, tetraploid amount, the cell will incorporate ^{3}H-thymidine into the DNA during this period. The cell then remains in interphase for a further period prior to the commencement of mitosis. This last compartment of interphase is called G_2, the cell being recognised as having 4c DNA content, but is not labelled with a flash dose of ^{3}H-thymidine. In the present study combinations of autoradiography and quantitative cytochemical methods have been used to investigate the relative distribution of the cell population in the compartments of interphase in Burkitt tumour biopsies.

MATERIALS AND METHODS

Biopsies were obtained from cases of Burkitt's tumour seen at the Kenyatta Hospital, Nairobi and Mulago Hospital, Kampala. A bone marrow specimen was also obtained from an European child (K.P.) aged 5, who was seen at the Hammersmith Hospital, London and diagnosed as Burkitt's tumour. This child had not lived in the tropics. In addition to the bone marrow involvement the disease involved her

liver, ovaries and a sub-mandibular lymph node.

Two experimental procedures were used in this study: first, an examination of the DNA contents of the nuclei of imprint preparations from the tumour biopsies and the bone marrow sample (K.P.).

The second technique used was to disperse the cells in 50: 50 autologous plasma and tissue culture medium TC199 (Glaxo). Tritiated thymidine (^{3}H-TdR), specific activity 1·9 c/m Mole, 2μc/ml, was added to the cell suspensions and samples were taken after 30 min or 5 hr at 37°C.

The cells were finally spread on slides, air dried and fixed with methanol.

Feulgen staining

The slides were placed in 1 N HCl for 10 min at 60°C for hydrolysis. This was followed by staining with Schiff's reagent for 1 hr at pH 1·6.

Microdensitometry

The DNA content of the cells was estimated by measuring the light absorption of the Feulgen stained nuclei at 5500Å using a Deeley pattern integrating microdensitometer [9].

Cell size measurement

The cell sizes of the bone marrow sample and of the ascites cells were measured using a photographic method [10].

Autoradiography

Feulgen stained slides were coated with Ilford K5 nuclear emulsion; the autoradiographs were developed after 4–8 days exposure. The compartment analyses were made by counting the frequency of the labelled cells (S compartment) in the 30-min incubation samples per 1000 cells. The relative numbers of cells in G_1 and G_2 were determined by measuring the DNA contents of 100 nuclei that were not labelled with ^{3}H-TdR. In some samples the distribution of the DNA contents of the cells known to be in S by their labelling with ^{3}HTdR at 30 min was studied by a technique that has been previously described [10].

The bone marrow sample (K.P.) and the ascites tumour cells were stained by the May Grünwald-Giemsa method and photographic maps prepared of representative areas of the slide. The Burkitt tumour cells were identified and their position recorded on the map, the stain was leached out of the preparation by dipping it in 5% trichloracetic acid and then placing in methanol for 5 min. After the dye had been removed the slide was stained by the Feulgen method.

RESULTS

Figure 1 shows a representative selection of histograms showing the distribution of DNA content in the nuclei of the Burkitt tumour cells in biopsy imprints. Additional metaphase

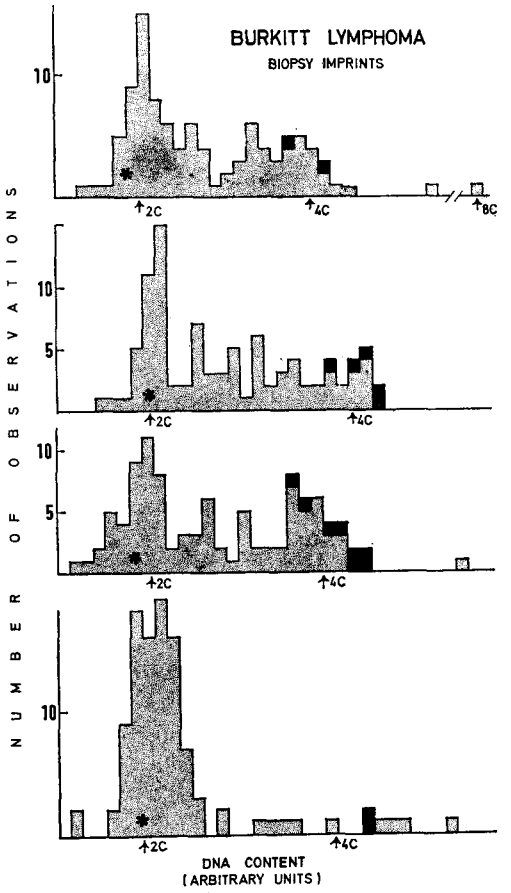

Fig. 1. Histograms of distribution of DNA contents in nuclei of Burkitt lymphoma cells. Imprit preparations.
**, 2c modal value for polymorphonuclear leucocytes.*
■, mitotic figure.

figures were measured in the samples so that an indication of the 4c mode could be obtained; hence the relative numbers of metaphase cells to interphase cells in some of the histograms is artificially high. It will be noticed that in Fig. 1 (i–iii) there is a large number of cells with DNA contents above the diploid mode, the majority of which can be considered to be in S or G_2. In Fig. 1 (iv), the cells were predominantly about the 2c modal value and there were only a few with DNA contents that indicated that they were engaged in DNA synthesis or were in G_2. Fifteen imprints were examined by this method : only one of them had the distribution pattern seen in Fig. 1 (iv), all the remainder had distribution patterns characteristic of a rapidly proliferating cell population.

Polymorphonuclear leucocyte nuclei, which were present in all the samples, were measured to obtain an estimate of the DNA content of normal resting diploid cells. The value obtained is probably 8–10% less than that of normal blast cells, due to the particular configuration of their nuclear chromatin [11].

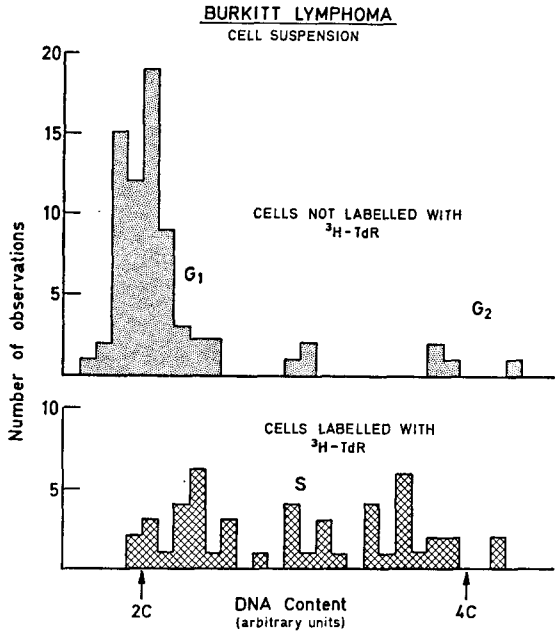

Fig. 2. Histogram of DNA contents of Burkitt lymphoma cells, showing the relation of the cells in G_1, S, and G_2.

The combination of autoradiography with microdensitometry gives further information about the distribution of cells in the various compartments of interphase. Figure 2 shows a detailed analysis of one of the populations of malignant cells obtained from a jaw tumour biopsy. In Table 1, interphase compartment distribution analyses are shown for twenty-one biopsy samples from children with Burkitt tumour. Patient No. 4 was treated with injections of irradiated tumour cells between the various times of biopsy. Patient No. 8 received a single dose of orthomerphalan, 38 mg i.v. and the second biopsy was taken 24 hr later. Patient No. 15 was critically ill when admitted to hospital and the biopsy was not taken until 2 weeks after his treatment with endoxan. In all these biopsy samples the distribution of DNA contents gave no indication of any marked aneuploidy, minor variations of chromosome number would not cause a large enough variation in DNA content to be detected by this method. Furthermore, relatively few cells were found with DNA contents indicative of being in the S compartment, i.e. between 2c and 4c modes that were not labelled with ^{3}H-TdR. This suggests that arrest in DNA synthesis was a rare event.

The relation between the amount of ^{3}H-TdR incorporated into the cells (grain count) and

Table 1. Compartment analysis of Burkitt tumour cells

Case No.	Age	Sex	Situ.	Percentage distribution of cells			Date
				G_1	S	G_2	
1	7	F	Jaw	56	38	6	
2	6	M	Jaw	50	45	5	
3	2	M	Jaw	53	38	9	
4a	6	M	Jaw	66	19	15	7.2.66
4b	6	M	Jaw	62	29	9	22.2.66
4c	6	M	Jaw	61	31	8	28.3.66
5	8	M	Jaw	59	32	9	
6	5	F	Jaw	88	10	2	
7	6	M	Jaw	67	20	3	
8a*	8	M	Jaw	74	14	8	28.3.66
8b†	8	M	Jaw	94	2	4	3.4.66
8c	8	M	Jaw	59	33	8	6.7.66
9	5	M	Humerus	58	37	5	
10	5	M	Jaws	86	12	2	
11	8	F	Abdominal mass	67	28	5	
11b	8	F	Ascites	63	29	8	
12	9	F	Abdominal mass	70	26	4	
13	10	M	Jaw	45	47	8	
14	4	M	Jaws	48	43	9	
15†	6	M	?Submandibular gland ?Cervical lymph nodes	56	34	10	
16			Jaw	46	47	7	

*Long past history. Maxillary tumour December 1964 treated with Cytoxan, November 1965 abdominal recurrence again treated with Cytoxan.
†After chemotherapy, see text for details.

their position in the S compartment (DNA content) when incubated with the isotope is shown in Fig. 3.

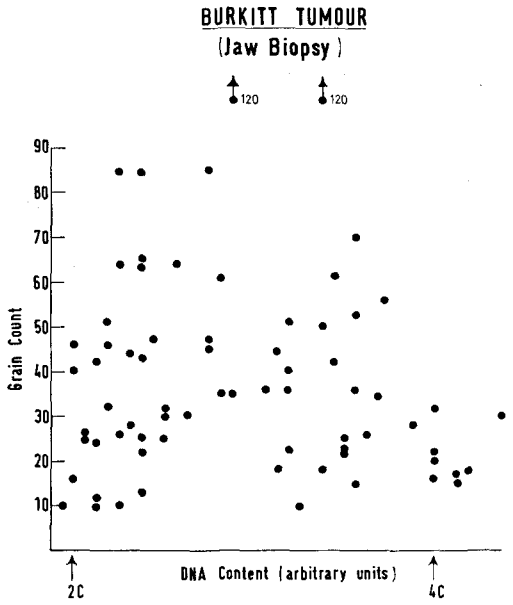

Fig. 3. Relation between incorporation of ^{3}H-TdR (grain count) and DNA content of the tumour cells, following a 30 min incorporation with ^{3}H-TdR.

The samples of the ascites cells, which came from a patient with a massive retroperitoneal tumour, may not be entirely typical of Burkitt's tumour as they were not growing as a solid tumour, and have the advantage that they can be studied with the minimum trauma during the preparation of the cell suspension. The relationship between the cell size, DNA content and interphase activity is shown in Fig. 4. In this sample the pyknotic cells were easily identified, measurement of their DNA content indicated that cell death probably occurred at any stage of the cell cycle.

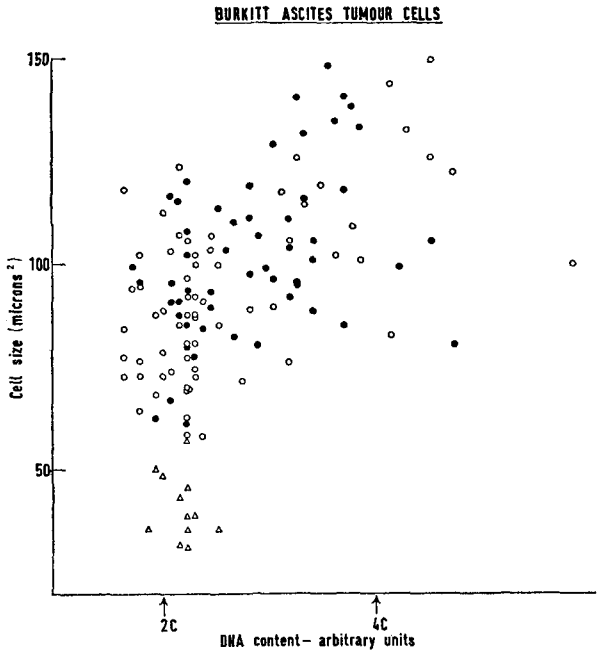

Fig. 4. Relation between cell size, DNA content and DNA synthetic activity in ascitic Burkitt tumour cells (30 min *incubation with ^{3}H-TdR*).

○, *tumour cell not synthesising DNA*
●, *tumour cell synthesising DNA* △, *small lymphocyte.*

The histogram of the DNA contents of the Burkitt tumour cells in the bone marrow of the patient K.P. is shown in Fig. 5, polymorphonuclear leucocytes and juvenile forms were used as a comparative cell line. The relation of the cell size to DNA content of the tumour cells is shown in Fig. 6.

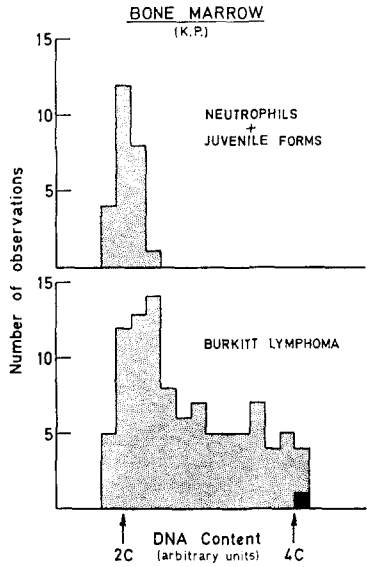

Fig. 5. Histogram of DNA content of cells in bone marrow of the patient K.P. ■, mitotic figure.

It was hoped that a comparison of the labelling index of the cells after 30 min incubation with ^{3}H-TdR with that after 5 hr would give an indication of the proliferation of the cells in the cultures. It can be seen, Table 2, that this

Table 2. Percentage labelling with ^{3}H-TdR after 30 min and 5 hr incubation

Case No.	30 min	5 hr
1	38	51
2	45	52
3	38	52
4	19	25
4b	29	34
7	20	22
5	32	40
6	10	11·5
11 ascites*	6·2	10·5

*This sample had a lower labelling index than 11b. It was probably due to a larger proportion of non-malignant cells in the ascitic fluid.

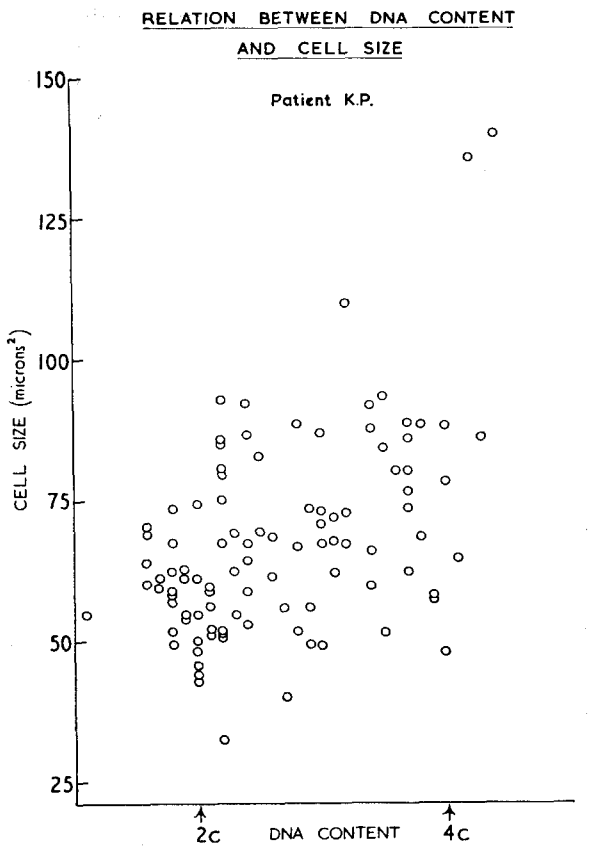

Fig. 6. Relation between cell size and DNA content of the Burkitt tumour cells in the bone marrow of patient K.P.

procedure gave very variable results which may be the result of trauma in preparing the cell samples.

DISCUSSION

There are several features of the proliferation of Burkitt's tumour cells that set this tumour apart from many other neoplasms in man. The very high percentage of the cells that are in DNA synthesis is unusual. Steel and Bensted [12] have calculated that the potential doubling times of tumours with a labelling of 34 per cent is 1 day. On this basis, seven of the eighteen biopsies from the patients who had been given no chemotherapy, had potential doubling times of less than 1 day and nine had potential doubling times of between 1 and 2 days. The term potential doubling time means, the rate of doubling of the tumour mass expected if each cell that commenced DNA synthesis were able to produce two viable daughter cells. However, these findings have to be contrasted with the clinical observation that, despite the fact that these tumours grow rapidly in comparison with many other malignant tumours, a rapidly growing Burkitt tumour usually takes a few weeks to double its size. It must be pointed out that these observations are made at a time when the disease is in an advanced state and there is little information about the initial rate of growth in Burkitt tumours.

In the patient No. 4, who was observed during an attempted treatment with immunotherapy, using irradiated tumour cells, between 19 and 31 per cent of the tumour cells were in S on the three occasions of biopsy, which were made over a 7-week period. During this time it was noted that the tumour underwent no change in size. The grain counts over the cells were equivalent to those found in normal tissues processed under the same conditions as the tumour cells, and where it was possible to make estimates, the mitotic index was of the order to be expected for the percentage of cells in S. These features suggested that the DNA synthetic period in the tumour cells was the same order of duration as has been reported for other cells with normal amounts of DNA i.e. between 6 and 10 hr [12, 13]. In a tissue culture strain of Burkitt's tumour cells EB_3 [4] with a normal chromosome number, the average duration of S was estimated to be 12 hr, but this may had been a little slower than *in vivo*, as the cells had been *in vitro* for 1 year.

The discrepancy between the potential doubling time and the actual rate of tumour growth must mean that either large numbers of cells are continually leaving the tumour or that there is a very high daily death rate of cells *in situ*. The facts that local lymph nodes draining a tumour mass are not commonly involved and that circulating Burkitt tumour cells in the peripheral blood are rare, are against the possibility that a large daily dissemination of tumour cells occurs in this disease. On the other hand, sections of the tumour almost always show a large number of non-neoplastic histiocytes scattered between the tumour cells. These histiocytes are frequently laden with pyknotic nuclei, cell debris, and sometimes even whole lymphoid cells.

Epstein and Herdson [13] first drew attention to possible degenerative changes in the nuclei of two biopsies from Burkitt's tumour. However, these may have in part been caused by long delays between biopsy and tissue fixation. Following a more extensive electron microscopic study of sixty-seven biopsies of this tumour in which rapid fixation techniques were used, Dourmashkin [14] noted that many of the cells were in various stages of degeneration. The close proximity of normal and degenerated cells suggested that the cell degeneration was a random process throughout the tumour and not the result of ischaemic necrosis. He could not

detect any evidence of virus particles in the degenerated cells. These observations all provide evidence suggestive of a high death rate of the tumour cells *in situ*. This supports the idea put forward by O'Connor [15] that the phagocytosis in the tumour is indicative of high rates of cell proliferation and cell death in the tumour. This type of histiocytic reaction is seen in other types of tumours, notably lymphosarcomata [16] but is also a feature in benign follicular reactive hyperplasia. The kinetics of this phagocytic process in the germinal centres of normal rats has indicated that there is a rapid turnover time of the tingible bodies (pyknotic nuclei) 2 days after a single injection of ^{3}H-TdR 48 per cent of the tingible bodies were labelled [17].

The high rate of cell proliferation and cell death contrasts with the relatively low rates of cell proliferation in acute leukaemia. In acute leukaemia the labelling index is frequently low and the main defect would be a maturation defect [18, 19].

The lack of direct correlation between the labelling index (potential doubling time) and the actual rate of increase of the tumour size contrasts markedly with that found in many experimental tumours. In transplantable tumours of rats and mice, the reproductive integrity of the tumour cells is very high and in several of these tumours there is a good agreement between the expected doubling time calculated from the duration of the S period and the percentage of cells in S and the observed rate of tumour growth (see review by Baserga [20]).

In the analysis of the Burkitt tumour cell populations, there were two compartments of interphase activity that could be recognised with certainty, S by virtue of the labelling with ^{3}H-TdR and G_2 from the characteristic DNA content of the cells. The remainder of the cells have been classified as being in G_1 (Table 1), but it must be pointed out that within this group there may be cells that are resting and can be considered to be out of cycle G_0 [21]. However, as yet there are no physical characteristics that enable us to distinguish a G_0 cell from a G_1 cell. In the majority of the specimens the higher labelling index would suggest that the bulk of the cell population was in cycle. In those few biopsies that had low labelling indices i.e. Fig. 1 (iv) and patient No. 11, ascites sample 1, there may have been a substantial number of cells in this state.

Treatment with alkylating agents has been reported to have produced some dramatic remissions and possible cures of Burkitt's tumour [22]. Patient No. 8 showed a marked inhibition of DNA synthesis 24 hr after a single dose of orthomelphalan but 30 days later this proliferation was once again restored to its high rate. Patient No 15 showed that a high rate of proliferation was still present 2 weeks after treatment with cyclophosphamide. This series of biopsies contains far too few samples to judge the effects of treatment at the cellular level. We are now extending this particular aspect of the research.

Diffuse involvement of the bone marrow is not commonly seen in cases of Burkitt's tumour in African children [15]. It is of interest to note that the same high rate of proliferation, as judged by the percentage of cells in S and G_2 of the tumour population, in the bone marrow of the patient K.P. and that this somewhat atypical tumour present in an European child did not show the proliferation characteristics of an acute leukaemia. In this case the cells varied in their size though maintaining their characteristic morphology. This variation did not appear to be simply related to the position of the cell in its cell cycle. In this later aspect the bone marrow tumour cells differed from that seen in the ascites cells where there was a good correlation between cell size and its position in interphase, indicating that there was considerable cell growth occurring between successive cell divisions.

The lack of correlation between grain count and DNA amount (position in the S phase) showed that there is no definite change in the rate of the DNA synthesis as the cell proceeds through the S period. This random pattern of grain count/DNA has been previously observed both in normal and leukaemic leucocytes [22].

Several workers have managed successfully to set up tissue culture lines from biopsy specimens of Burkitt tumours [5, 6]. Table 2 shows that there was a very considerable difference in the ability of the various cell samples to maintain their proliferation *in vitro*. Those in which a definite increase in the number of cells that were labelled with ^{3}H-TdR in the 5 hr sample as compared to the 30 min sample, can be assumed to have labelled cells proceeding through G_2 and division and also new cells entering S during the incubation period. In others there was little evidence of sustained proliferative activity. It is unlikely that the initial estimates of the numbers of cells in S at the time of taking the samples are false, since examination of the DNA contents of the nuclei not labelled with ^{3}H-TdR showed them in the main to be either in the G_1 or G_2 compartments. If a marked inhibition of DNA synthesis

occurred shortly after taking the samples, then many unlabelled cells would be expected to have DNA contents lying between the 2c and 4c modes; this was only found infrequently. It was initially hoped that the study of the proliferation of the cells *in vitro* might reveal information about their kinetic behaviour, however, it is now realised that arrest of progress through the cell cycle may readily occur in these cells *in vitro*, a problem that has been previously met during the study of the proliferation of infectious mononucleosis lymphocytes *in vitro* [24]. Hence it is now considered that a long period of labelling *in vitro* may result in severe perturbation of the normal cell kinetics and lead to rather misleading results.

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RÉSUMÉ

Des biopsies de tumeur de Burkitt ont été examinées par les méthodes cytochimique et autoradiographique quantitatives. L'incorporation de thymidine H^3, après une incubation de 30 minutes, montra que, dans 17 biopsies de malades non traités, des temps de doublement potentiels de moins d'un jour furent trouvés dans 39% des cas, et de moins de deux jours dans 88% d'entre eux. La mesure de la charge en DNA des cellules individuelles confirma la forte activité proliférative de la population cellulaire, mais ne démontra pas d'aneuploïdie marquée, non plus qu'un arrêt des cellules en période S. Les relations entre la charge en DNA, la dimension de la cellule, et la captation de thymidine H^3, ont été examinées. On avance l'hypothèse que cette tumeur montre généralement une forte prolifération cellulaire, associée à un taux de mortalité cellulaire élevé. La phagocytose des cellules en picnose par les histiocytes, particularité classique de la tumeur, paraît être favorisée par un taux de mortalité cellulaire élevé.

SUMMARY

Biopsies of Burkitt's Tumours have been examined by quantitative cytochemical and autoradiographic methods. Incorporation of ^{3}H-thymidine after 30 min incubation, indicated that in seventeen biopsies from untreated patients, potential doubling times of less than 1 day were found in 39 per cent and less than 2 days in 88 per cent. Measurement of the DNA content of individual cells confirmed the high proliferative activity in the cell populations, but failed to demonstrate any marked aneuploidy or arrest of cells in the S period. Relations between DNA content, cell size and ^{3}H-thymidine uptake have been examined. It is proposed that this tumour usually has a cell high proliferation rate which is associated with a high cell death rate. Phagocytosis of pyknotic cells by histiocytes, a classical feature of the tumour, is thought to be promoted by a high tumour cell death rate.

ZUSAMMENFASSUNG

Burkitt-Tumoren-Biopsien wurden durch quantitative cytochemische und autoradiographische Methoden geprüft. H^3-markiertes Thymidin, nach einer Incubation von 30 Minuten zugesetzt, zeigte dass in 17 Biopsien von unbehandelten Kranken potentielle Verdoppelungszeiten in 39% der Fälle weniger als einen Tag betrugen während in 88% der Fälle Werte von weniger als zwei Tage gefunden wurden. Durch das Messen des DNS-Gehaltes von einzelnen Zellen wurde die hoch proliferative Wirkung in der Zellenpopulation bestätigt; dadurch wurden aber weder eine deutliche Aneuploidie noch ein Stillstand der Zellen während der S-Periode nicht nachgewiesen. Die Beziehung zwischen dem DNS-Gehalt, dem Zellenmass und der Aufnahme von H -markierten Thymidin wurde geprüft. Es wird vermutet, dass dieser Tumor gewöhnlich ein starkes Zellenwachstum zeigt, welches mit einer hohen Zellensterblichkeit verbunden ist. Es scheint, dass die als klassische Eigenschaft des Tumors beschriebene Phagocytose der pyknotischen Zellen durch die Histiocyten, durch die hohe Sterblichkeitsrate der Geschwulstzellen bedingt sei.

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Letter to the Editor

Ibenzmethyzin (Natulan®), a Highly Effective Mammary Carcinogen in the Huggins System*

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HUGGINS and coworkers developed a remarkable method for the rapid induction of hormone-dependent mammary cancers in the albino rat. A single gastric or intravenous dose of 7,12-dimethylbenz(a) anthracene (712-DMBA) evoked multiple mammary cancers in all animals when given under certain specific conditions, namely to female rats of the Sprague-Dawley strain near the age of 50 days [1]. Dividing the dose in three successive injections at age 50, 53 and 56 days resulted in a still higher yield of tumors [2]. Other polycyclic aromatic hydrocarbons were also effective, provided they fulfilled physico-chemical criteria formulated by Huggins [3].

The purpose of the experiment related here was to determine whether this particular type of mammary carcinogenesis could be duplicated by carcinogens other than the polycyclic aromatic hydrocarbons. Ibenzmethyzin†, a water-soluble methylhydrazine derivative used as an effective agent in human cancer chemotherapy, was selected for study because it was reported to induce mammary tumors in Osborne-Mendel rats when repeatedly administered since birth [4]. In the present experiment, young female rats of the Sprague-Dawley strain were used under the precise conditions described by Huggins and referred to herein as the Huggins system. Ibenzmethyzin in aqueous solution was administered by gastric intubation to four groups of ten rats. A fifth group serving as control received 7,12-DMBA dissolved in sesame oil.

Table 1. *Production of mammary tumors by ibenzmethyzin*

No. of rats	Compound	No. doses	Total dose (mg)	No. of tumor-bearing/ no. of surviving rats		Active centers mean
				Weeks after treatment		
				10	20	
10	Ibenzmethyzin	1	50	5/10	8/8	2·9
10	Ibenzmethyzin	1	100	2/5	5/5	6·0
10	Ibenzmethyzin	1	150	0/2	2/2	3·5
10	Ibenzmethyzin	3	150	1/5	5/5	4·4
10	7,12-DMBA	3	20	6/9	7/7	3·3

(Female rats were fed the carcinogen either as a single dose at age 50 days or as three successive doses at age 50, 53 and 56 days.)

The doses, dosage schedules and results are shown in Table 1. All surviving animals developed mammary tumors within 20 weeks. A single oral dose of 50 mg of ibenzmethyzin proved as effective as 7,12-DMBA. Higher doses were poorly tolerated. In order to determine whether these tumors were hormone-dependent, six rats bearing a total of seventeen

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†Ibenzmethyzin = p-(N^1-methylhydrazinomethyl)-N-isopropyl benzamide hydrochloride; Natulan (Trade Name) was kindly supplied by Roche Products S. A., Bruxelles.

tumors were oophorectomized 21 weeks after one single dose of 50 mg of ibenzmethyzin. All tumors except one disappeared within 6 weeks following oophorectomy. Autopsy was performed on the seventeen rats surviving 6 months after ibenzmethyzin administration. In the oophorectomized group, the single non-regressed tumor was a fibro-adenoma. In the 11 non-oophorectomized rats, the mammary tumors comprised thirty-nine adenocarcinomas, undistinguishable from those induced by 7,12-DMBA, and one fibroadenoma. In addition, postmortem examination disclosed five squamous cell carcinomas of the ear duct and one renal sarcoma. Adrenal calcification, as observed after 7,12-DMBA administration [5], was not found in these rats.

These results indicate that mammary carcinogenicity in the Huggins system is not restricted to polycyclic aromatic hydrocarbons, since a non-polycyclic compound, ibenzmethyzin, was found equally effective. Similar conclusions were reached independently by Griswold *et al.* in a recent report [6]. These authors, studying the carcinogenicity of fifty compounds in the same experimental system, demonstrated carcinogenic activity in five compounds that are not polycyclic aromatic derivatives. However, the most active of these compounds, [N-6-(3,4-benzocoumarinyl) acetamide, evoked mammary cancer in only 30 per cent of the treated rats, whereas by ibenzmethyzin the yield was 100 per cent. It is concluded that by the method of Huggins ibenzmethyzin apparently is by far the most potent mammary carcinogen among the non-polycyclic compounds studied as yet and that it is as effective as the most potent polycyclic aromatic hydrocarbon, 7,12-DMBA.

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LES PROBLÈMES liés au diagnostic et au traitement des tumeurs malignes de la glande thyroïde continuent à être l'objet de controverses.

Dans l'espoir de résoudre certains de ces problèmes, le Groupement Coopérateur de Radiobiologie Clinique a créé un Registre du Cancer Thyroïdien.

Il est souhaitable que le plus grand nombre de nouveaux cas de cancer de la thyroïde, pris en traitement ou diagnostiqués, soient enregistrés. Pour cela, il y a lieu d'adresser au Secrétaire du Registre un résumé de l'observation, sous forme de réponse à un questionnaire, des lames histologiques caractéristiques et tous autres documents utiles.

Les médecins désireux de collaborer à ce travail sont invités à se mettre en rapport avec le Secrétaire du Registre. Un exemplaire du questionnaire leur sera adressé ultérieurement.

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Beginning with No. 3, the European Journal of Cancer will accept "letters to the Editor" for prompt publication. The length of these short communications should not exceed 750 words including title, authors, bibliography and acknowledgements. They should furnish sufficient data to support their conclusions and conform to the usual standards of the Journal.

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